THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

FRANK A. BROWN, JR., Northwestern University
E. NEWTON HARVEY, Princeton University
M. H. JACOBS, University of Pennsylvania
DANIEL MAZIA, University of California
C. LADD PROSSER, University of Illinois
A. GLENN RICHARDS, University of Minnesota
F. SCHRADER, Columbia University
H. B. STEINBACH, University of Minnesota
WM. RANDOLPH TAYLOR, University of Michigan
ALBERT TYLER, California Institute of Technology
DOUGLAS WHITAKER, Stanford University
RALPH WICHTERMAN, Temple University

DONALD P. COSTELLO, University of North Carolina
Managing Editor

VOLUME 100
FEBRUARY TO JUNE, 1951

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $1.75. Subscription per volume (three issues), $4.50.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 15 and September 1, and to Dr. Donald P. Costello, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina, during the remainder of the year.

Sci.
QH
301
B59
vol. 100-101
1951

Entered as second-class matter May 17, 1930, at the post office at Lancaster, Pa., under the Act of August 24, 1912.

LANCASTER PRESS, INC., LANCASTER, PA

General
Exch

CONTENTS

No. 1. February, 1951

PAGE

Butros, Joseph
The action of saponin and its non-hemolytic modification on growth..... 1
Chadwick, Leigh E.
Stroke amplitude as a function of air density in the flight of Drosophila.. 15
Enami, Masashi
The sources and activities of two chromatophorotropic hormones in crabs of the genus Sesarma. I. Experimental analyses..... 28
Leone, Charles A.
A serological analysis of the systematic relationship of the brachyuran crab Geryon quinquedens..... 44
Rudzinska, Maria A., and Robert Chambers
The activity of the contractile vacuole in a suctorian (Tokophrya infusionum)..... 49
Fauré-Fremiet, E.
The marine sand-dwelling ciliates of Cape Cod..... 59

No. 2. April, 1951

Davenport, Demorest, and John F. Hickok
Studies in the physiology of commensalism. 2. The polynoid genera Arctonoë and Halosydna..... 71
Dolley, William L., Jr., and John D. White
The effect of illuminance on the reversal temperature in the drone fly, Eristalis tenax..... 84
Dolley, William L., Jr., and John D. White
The effect of illuminance on the lethal temperature in the drone fly, Eristalis tenax..... 90
Roeder, Kenneth D.
Movements of the thorax and potential changes in the thoracic muscles of insects during flight..... 95
Sedar, Albert W., and Donald F. Wilson
Electron microscope studies on the normal and colchicinized mitotic figures of the onion root tip, Allium cepa..... 107
Weisz, Paul B.
Homoplastic grafting in Stentor coeruleus..... 116
Lehman, Harvey Eugene
An analysis of the dynamic factors responsible for the phenomenon of pigment suppression in salamander larvae..... 127

No. 3. JUNE, 1951

BODINE, JOSEPH HALL, AND KIAO-HUNG LU
Endogenous oxygen uptake and specificity of embryonic intracellular constituents 153
COOPERSTEIN, S. J., AND ARNOLD LAZAROW
Cytochrome oxidase and succinic dehydrogenase content of squid (Loligo pealii) nervous tissue 159
ELLIOTT, ALFRED M., AND ROBERT L. HUNTER
Phosphatase activity in Tetrahymena 165
FAURÉ-FREMIET, E.
The tidal rhythm of the diatom Hantzschia amphioxys 173
HUGHES-SCHRADER, SALLY
The desoxyribonucleic acid content of the nucleus as a cytotaxonomic character in mantids (Orthoptera: Mantoidea) 178
KELLY, SALLY, AND NORMAN A. BAILY
The uptake of radioactive iodine by Ascophyllum 188
LAZAROW, ARNOLD, AND S. J. COOPERSTEIN
Studies on the isolated islet tissue of fish. I. The cytochrome oxidase and succinic dehydrogenase contents of normal toadfish (Opsanus tau) .. 191
MEHLMAN, BENJAMIN, AND THEODOR VON BRAND
Further studies on the anaerobic metabolism of some fresh water snails .. 199
PIERCE, E. LOWE
The Chaetognatha of the west coast of Florida 206
SACKTOR, BERTRAM
Some aspects of respiratory metabolism during metamorphosis of normal and DDT-resistant house flies, Musca domestica L. 229
SARBAHI, DAYA SHANKAR
Studies of the digestive tracts and the digestive enzymes of the goldfish, Carassius auratus (Linnaeus) and the largemouth black bass, Micropterus salmoides (Lacépède) 244

UNIVERSITY
OF MICHIGAN
MAR 7 1951
SCIENCE
LIBRARY

Volume 100 Number 1

THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

FRANK A. BROWN, JR., Northwestern University
E. NEWTON HARVEY, Princeton University
M. H. JACOBS, University of Pennsylvania
DANIEL MAZIA, University of Missouri
C. LADD PROSSER, University of Illinois
A. GLENN RICHARDS, University of Minnesota
F. SCHRADER, Columbia University
H. B. STEINBACH, University of Minnesota
WILLIAM RANDOLPH TAYLOR, University of Michigan
ALBERT TYLER, California Institute of Technology
DOUGLAS WHITAKER, Stanford University
RALPH WICHTERMAN, Temple University

DONALD P. COSTELLO, University of North Carolina
Managing Editor

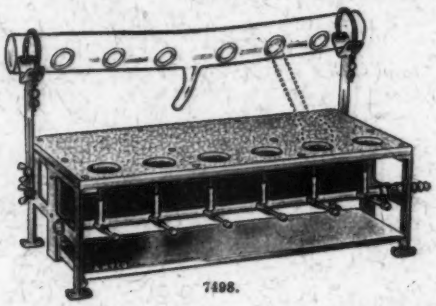
FEBRUARY, 1951

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

A. H. T. CO. SPECIFICATION

MICRO KJELDAHL DIGESTING APPARATUS

With improved type fume duct of Pyrex glass, metal shield behind the burners to protect the flames from drafts, and metal shelf below the burners to minimize heat transfer to table top



7498.

KJELDAHL DIGESTING APPARATUS, MICRO, A.H.T. Co. Specification, for gas heating. Suitable for either micro or semimicro analysis as it takes Kjeldahl flasks 10 ml, 30 ml or 100 ml capacity, the latter if finished without rim at neck.

Consisting of a six-burner heating shelf with transite top and a fume duct made of Pyrex brand glass. Heating shelf is made of cast brass with baked-on aluminum finish, with metal shield behind the burner manifold to protect the flame from drafts, and metal shelf below the burner manifold to deflect heat upward, thereby minimizing heat transfer to table top. Top is 18¼ inches long × 8½ inches wide × ¼-inch thick, with six tapered burner openings, 1⁹⁄₁₆ inches diameter at top × 1 inch diameter at bottom, 3 inches from center to center, each fitted with concave wire gauze which concentrates rather than diffuses heat from the burners. Burners are regulated by individual needle valves conveniently located in front of each burner.

The fume duct is made of heavy wall Pyrex brand glass, 516 mm long × 51 mm outside diameter, with six openings each 22 mm diameter for insertion of flask necks. The fume duct is provided with a central outlet and is sloped toward the center to facilitate drainage in use and to assure equal suction at both ends, as suggested by Eugene W. Schoeffel, while with the American Medical Association Laboratories, Chicago, Ill. The fume duct is held in position by means of two adjustable, slotted aluminum clamps with spiral springs for variation in tensity.

7498. **Kjeldahl Digesting Apparatus, Micro, A.H.T. Co. Specification,** as above described, for use with artificial or mixed gases up to 600 B.T.U. Complete with fume duct made of Pyrex brand glass, two 5039-C Clamps to support duct and six concave discs of Chromel wire gauze to fit burner openings, but without flasks or rubber connections **72.60**

ARTHUR H. THOMAS COMPANY

RETAIL—WHOLESALE—EXPORT

LABORATORY APPARATUS AND REAGENTS

WEST WASHINGTON SQUARE PHILADELPHIA 5, PA., U. S. A.

Cable Address, "BALANCE," Philadelphia

Vol. 100, No. 1 February, 1951

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE ACTION OF SAPONIN AND ITS NON-HEMOLYTIC MODIFICATION ON GROWTH[1]

JOSEPH M. BUTROS

Biology Department, American University of Beirut, Beirut, Lebanese Republic

The hemolytic activity of saponins has been extensively studied and has stimulated investigations related to other properties of these compounds. A series of studies on seed germination and plant growth are reported by Balansard and Pellissier (1943; 1944; 1945) and indicate a growth-promoting activity of saponin in very dilute solutions (10 to 100 ppm.). Such solutions produce considerable proliferation forming tumor-like concrescences in ivy shoots (1943). Seedlings from treated cereal seeds grow faster and become larger than their controls (1944). Similarly the germination of tomato seeds was accelerated (1945). Dilute saponins accelerate water absorption of moistened seeds and speed up germination of corn (Balansard, Pellissier and Conil, 1946).

On the other hand, the inhibitory action of saponins in higher concentration has been reported by some authors. Amerio and Dalla (1942) found that the roots of *Allium cepa* and coleoptiles of oat were irreversibly but non-specifically damaged. Von Euler (1946) found that seedlings of *Lepidium sativum* and *Hordeum vulgare* grew to only one-third to one-tenth of the lengths of the controls. Inhibitory action is also reported in animals: heart and lung tissue of hen embryo (Hideo, 1928) and Jensen sarcoma (Frey, 1938).

Two years ago the author described the cytolytic effects of 0.5 per cent Merck saponin on rat tissue and tumors (Butros, 1948). The Feulgen stain showed degenerative changes in the nucleus. This suggested a nucleo-toxic action and the necessity of a study of the effects of lower concentrations, especially on growing tissues. As onions have often been used in the study of mitosis and growth, they were here also employed in the greater part of these experiments.

Another aspect of saponin action was investigated also, *i.e.*, the possibility that its nucleo-toxic action is independent and separate from its hemolytic action. Hemolysis can be inhibited by a combination of saponin with cholesterol (as will be discussed further on). The nuclear damage might be another matter, and might depend on the action of saponin on some constituent other than those involved in hemolysis. If so, its action might show itself by interfering with mitosis and growth even if it has lost its hemolytic power. After sufficient indications of inhibitory action of both saponin and its "cholesteride" were evident by trials with onion roots,

[1] Supported by a grant from the Research Committee of the School of Arts and Sciences of the University.

the author started investigations on animals. The toxicity of the "saponin cholesteride" was much less than saponin of the same concentration and so it was felt safe enough to test it on tadpole development. Experiments with a non-hemolytic saponin-eosin preparation are also described.

The Saponins Used

Two saponins have been used in this work: Merck's (white pure), and Baker's (yellow). Solutions were made in tap water for the onion roots, in distilled water for the others.

The hemolytic strength of these two saponins was not the same. Hemolytic tests in this paper refer to the standard method of Ponder (1948). The time of complete hemolysis is the one considered except in some cases, especially of "saponin cholesteride," where an approximation of the percentage of hemolyzed r.b.c. by the microscope was employed.

Baker saponin in 1/1000 dilution resulted in complete hemolysis in three minutes. A 1/10,000 solution needed 18 hours to complete hemolysis, but a solution of 1/15,000 brought about only 75 per cent hemolysis in the same length of time. Merck saponin solutions of 1/50,000 show complete hemolysis in 18 hours; 1/100,000 show 50 per cent hemolysis in 18 hours and 1/200,000 show 25 per cent hemolysis in the same time.

Action of Saponin on Onion Root Growth

Onions (*Allium cepa*) were sprouted in water before placing them in the saponin solutions. Whole and half onions were used, the sister halves of the treated being used as controls in water. Measurements of root lengths were made and sample roots were fixed for cytologic preparations. Roots that had stopped growth in saponin were returned to water to determine their ability to resume growth. Very dilute solutions of saponins were included in this study to compare with the work of Balansard and Pellissier referred to in the introduction.

Inhibitory Concentrations

An arrest of root lengthening was definitely established within one or two days, depending on individual susceptibility, after placement in 0.05 per cent to 0.1 per cent Merck saponin and 0.25 per cent to 0.3 per cent Baker saponin. If roots were returned to water at this point, most of them were able to resume growth, but if left more than four days in the saponins they could not. The new growth was easily distinguished by its fresh, white texture as compared with the brownish treated tips. There was often a knot-like hypertrophy at the junction between the area grown in saponin and that grown in water. Curvature of the roots was noted in saponin but not in the controls. If left in the solutions over a week, the arrested roots developed secondary roots that came to a standstill in a couple of days. New roots from the periphery of the onion sometimes developed (Fig. 1).

From the study of sections taken at intervals of 12 hours for five days in saponin, it became clear that the mitotic process was inhibited completely by the second day, when no mitotic process was found. All cells were in the resting stage or in what appeared as very early prophase (Fig. 2). The chromatin particles were small,

thin rods. No disturbances or aberrations were noticed in the mitotic figures of the first day except that they were somewhat less numerous than the control. The region of elongation was not modified. Comparisons between microscopic and length studies were in agreement; roots that resumed lengthening after return to water showed recurrence of normal mitoses as abundant as the controls.

FIGURE 1. Onion that was replaced in water after treatment with 0.1 per cent Merck saponin, showing arrested roots with knotty beaded tips and secondary growth.

Lethal Concentrations

If roots were left long enough (4 to 5 days) in an inhibitory concentration, or for two days in a higher concentration, cytolytic degeneration occurred, ending in death of the desiccated roots. The nuclei became first pycnotic, then shrunken, pointed and eccentric. The cytoplasm gradually vacuolated out. Cells at the root cap were also displaced and disintegrated.

Sub-Inhibitory Concentrations

Experiments were done as described above: half onions were placed in the saponin solutions and their control halves in water. The length that represented

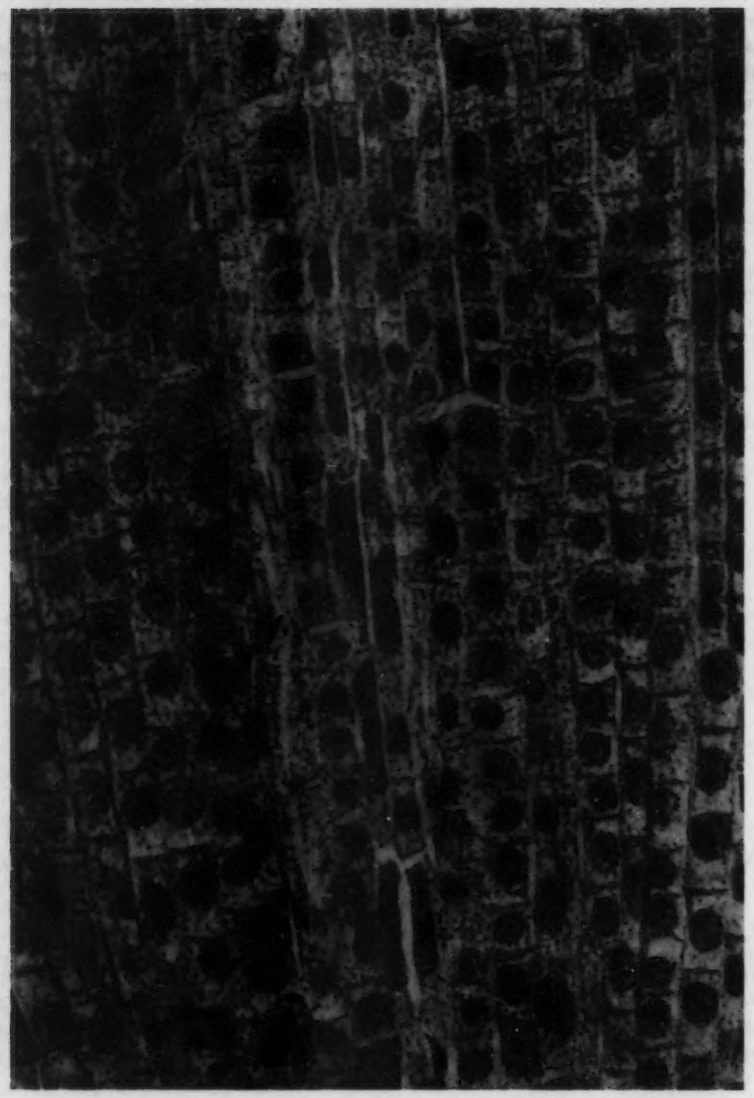


FIGURE 2. Section of onion root showing inhibition of mitosis in 0.1 per cent Merck saponin; fixed in Bouin's and stained with hematoxylin and eosin. × 400.

the majority of the roots in each specimen was tabulated daily, from which increments were calculated. The concentrations used were:

Merck: 1/5000; 1/10,000; 1/100,000; 1/500,000
Baker: 1/1000; 1/2000; 1/10,000; 1/500,000; 1/1,000,000; 1/2,000,000

As a summary, Table I gives the length measurements and compares the growth rates of the various concentrations of Baker saponin.

For a check on the deviation between normal onion halves an experiment was run with onion halves in two sets of vessels, both containing water. The average difference in length between the two sets at the end of 12 days was 0.34 cm. This value was used in evaluating the significance of experimental results.

TABLE I

Growth of onion roots in various concentrations of Baker saponin

No. of Days in Solution	Concentration	Length in Saponin	Length in Water	Increase in Saponin	Increase in Water	Growth Index*
7	1/2,000,000	9.9 cm.	8.9 cm.	8.9 cm.	7.9 cm.	1.1
10	1/500,000	11 cm.	9.3 cm.	10 cm.	8.3 cm.	1.2
6	1/10,000	8.2 cm.	9.4 cm.	7.2 cm.	8.4 cm.	0.9
8	1/2000	7.1 cm.	9.4 cm.	6.1 cm.	8.4 cm.	0.71
5	1/1000	5.1 cm.	8.4 cm.	4.1 cm.	7.4 cm.	0.53

* Increment per day in saponin divided by increment per day in water.

On examining Table I it will be seen that the very dilute concentrations of Baker saponin may be considered to favor growth. This agrees with the findings of Balansard and Pellissier referred to above. The lowest dilutions of Merck saponin used in these experiments did not show this growth-promoting power. As Merck saponin is stronger, they really compare to higher concentrations in terms of Baker saponin, which are also non-growth promoting.

Microscopic examination of root sections treated with the sub-inhibitory solutions of saponin showed apparently normal mitotic figures and healthy cells. The sub-inhibitory dose, however, is capable of producing inhibition of mitosis and cytolysis of cells if the roots were left long enough in the solution. After 6 days in 1/10,000 Merck saponin, the cells were vacuolated and no mitotic figures were discernible.

The Respiration of Onion Roots in Saponin

Trials were made to find out if saponin suppressed the respiration of onion roots. If it did, its inhibitory action could have been due to, or at least related to such action. The indicator bromthymol blue was used in most cases. This was very reliable as shown by repeated trials with growing roots, roots killed in alcohol, and roots immersed in KCN. It was found that in 0.3 per cent Baker saponin (which is sufficient to stop mitosis in two days), the roots were able to decolorize bromthymol blue, indicating production of an acid; pH measurements of the solutions containing the onions served as a further check on the production of carbon dioxide. The pH of the saponin solution in which onions grew was 6.4. Its control with no onion was

7.4, and a second control with a dead onion was 7.6. Further, distilled water containing respiring onions had a pH of 5.4 on the second day; its control with no onion was 6.0. These results show that onion roots continue to respire in saponin while their mitotic process is gradually coming to a standstill.

Preparation of Non-Hemolytic "Saponin Cholesterides"

In the extensive article on saponins by Kobert-Rostock (1924), it is explained that all saponins lose their hemolytic activity when their solutions are warmed with cholesterol. Windaus (1909) made a study of this reaction by using a steroid saponin, digitonin, which, although different in structure from the ordinary saponins, shares in their hemolytic activity. He made a digitonin cholesteride compound by precipitating it from an alcoholic solution of the two compounds. The cholesteride is not separated into its constituents by ether but is separated by boiling xylene. Using ordinary saponin, Meyerstein (1910) rendered it non-hemolytic by shaking it with cholesterol in normal saline. He stated that it is not of great importance whether these two compounds were held up by a genuine chemical combination or only by adsorption. His preparations were freshly made when needed.

The author used a modification of Meyerstein's method, which gave a stable non-hemolytic suspension that could stand over two months without splitting back into saponin and cholesterol. The method depended upon the use of a homogenizer [2] which ground the cholesterol into very fine particles. This was run into hot saponin solution and the reaction (or adsorption) took place instantaneously, as shown by the loss of frothing and loss of hemolytic action. Comparison with hemolytic tests made on saponin was very helpful in determining approximately the amount, if any, of free saponin in the suspension, and so indicating the possibility that the biological action might be due to free saponin.

A phosphate buffer was sometimes added to the "saponin cholesteride" when it was used with tadpoles. This seemed to favor the adsorption of the two components and make the compound more stable. The proportions of the components used were:

Baker saponin 3 gm. per liter; cholesterol 1.5 gm. per liter
Merck saponin 3 gm. per liter; cholesterol 2.5 gm. per liter

Saponin did not become non-hemolytic except when there was an excess of cholesterol. The suspension remained milky and non-hemolytic for over two months.

Properties of the "Saponin Cholesteride"

If the suspension is prepared successfully, it does not froth, is milky with the larger particles settling down, but it does not clarify even within three months. Merck's preparation is white, and Baker's light brown. Sometimes, for unknown reasons, it was not possible to make the suspension as there was a flocculation. The clear fluid, however, of the flocculated preparation was non-hemolytic and had the same inhibitory property as the milky suspension.

The clear filtrate of the "saponin cholesteride" remained non-hemolytic for a few days during which it was inhibitory to onion roots like the mother compound.

[2] The author is deeply indebted to Messrs. Awad and Baramki of the Chemistry Department for their help in this matter.

The color of the filtrate of the Baker preparation was pinkish, whereas the color of solutions of this saponin was yellow and never pink, no matter how dilute. The clear filtrate does not froth also, another indication that it does not contain free saponin. It could have been a saponin-cholesterol complex.

Extraction with ether of the Merck cholesteride until all excess cholesterol was removed gave a milky suspension with no microscopically visible crystals of cholesterol. This extract was non-hemolytic also (after evaporation of the hemolytic ether), but it became hemolytic on standing a few days, possibly due to the lack of excess cholesterol.

Thus it seems that the "saponin cholesteride" prepared in this manner was of variable and uncertain composition, and deserves a detailed study.

Action of "Saponin Cholesteride" on Onion Roots

Whole and half onions were placed in the suspension; controls were in water and in suspended cholesterol. In all cases, concentrations refer to the amount of saponin in the suspension.

Results in Baker Saponin

A 1.8 per cent suspension checks the growth in length of the roots in two days. The tips have the knotty appearance which was noted in saponin, and they curve upwards. A 0.9 per cent suspension was also able to check the growth of the roots within two days. Microscopic examination revealed the absence of mitotic figures, all cells being in the resting stage. Signs of beginning degeneration were apparent on the third day, indicated by a very little vacuolation of the cytoplasm and slight shrinkage of nuclei. A few roots with more resistance showed four or five mitotic figures per field ($\times$ 250). In the 0.3 per cent suspension, mitotic figures were found in the slides but about 10 per cent less than in the control.

Results with 0.3 Per Cent Merck "Saponin Cholesteride"

This concentration was insufficient to stop growth and mitosis completely. However, there was a 50 per cent reduction in the number of mitoses as compared with water control, and with dilute saponin control. The rate of lengthening dropped to 30 per cent. The roots were thicker than usual and the microscopic view indicated hypertrophy of the cells. It can be assumed from trials with Baker saponin that if the concentration were raised, complete arrest would result.

Proof That the Action is Not Due to Free Saponin

The hemolytic power of various concentrations of the "saponin cholesteride" was determined to help in approximating the amount, if any, of free saponin in the cholesteride. It should be remembered that complete hemolysis occurs within 18 hours in a concentration of 1/10,000 of Baker saponin but not if the concentration is 1/15,000 or 1/20,000. As the concentration of saponin required to stop mitosis is 0.3 per cent, which causes hemolysis in a few minutes, and as the "saponin cholesteride" did not show complete hemolysis in a much longer period (24 hours), there is no possibility of its action being due to saponin. Similarly, in the case of Merck saponin, there is immediate complete hemolysis in up to one per cent. The mitotic inhibitory dose is within this range, and as there was no complete hemolysis of the

"cholesteride" overnight, it is out of the question that the inhibition (partial in this case) was due to free saponin.

Daily hemolytic tests were made from samples of "saponin cholesteride" in which onions were growing, to eliminate the possibility that any saponin was splitting from the cholesteride as a result of the action of some agent from the onion. No splitting took place when only the roots were immersed in the suspension.

Filtered Merck "saponin cholesteride" (0.3 per cent) did not cause complete hemolysis in 24 hours. It arrested the growth of onion roots after a growth of ½ cm. during the first day.

Controls

Onions were grown in cholesterol suspension made by the homogenizer of the same strength as the 0.3 per cent cholesteride (which is partially inhibitory). The rate of growth in length was as follows:

In water	1 cm. per day
In cholesterol	0.8 cm. per day
In "saponin cholesteride"	0.4 cm. per day

In another experiment, corresponding half onions were placed in cholesterol and water respectively. The cholesterol in this case was ground in a mortar and then shaken several hours by a shaking machine to the same strength as the "saponin cholesteride." There was no apparent difference between the length of roots in cholesterol with those of the water.

Curvature of Onion Roots

Root curvature in saponin and its "cholesteride" suggested interference with auxins, although the latter are concerned with root sprouting rather than with root growth (Went and Thimann, 1937). If the "cholesteride" interferes with auxin action, then it might prevent the sprouting of roots, so onions were placed in these solutions. In both saponin and the "cholesteride," the onion roots appeared and grew for two to three days. However, they stopped at the length of 1.5 cm., as they usually do if placed after sprouting. It does not seem that curvature in these solutions had a relation to auxin action.

Toxicity of "Saponin Cholesteride" to Animals

A comparison of the toxicity of saponin and its "cholesteride" of the same concentration was made by noting their killing time on certain animals. In general the saponin toxicity was greatly decreased. For example, paramecium is cytolyzed instantaneously in 0.45 per cent Baker saponin, whereas it continued to move about for 5 hours in double that concentration of "cholesteride."

Table II compares the duration of life in Baker saponin and its "cholesteride" for the fresh water snail *Melanopsis bovieri* and planaria.

Toxicity studies were also made on tadpoles, and it was clear that the toxicity was greatly reduced when compared to that of pure saponin. It took 12 hours to kill tadpoles in 0.9 per cent "saponin cholesteride," whereas saponin of that concentration killed them in a few minutes. The "cholesteride" was non-hemolytic and this property was tested daily and in particular during the interval that the tadpoles were

in the suspension. This is significant as it points to a toxic property independent and separate from the known hemolytic property.

Filtered Merck "cholesteride" (0.2 per cent) kills tadpoles in 5 hours and shows complete hemolysis in less than 20 hours at 37° C. At room temperature it does not show complete hemolysis in that time, however. The ether-extracted

TABLE II

Duration of life in Baker saponin of Planaria and snails

Concentration	Saponin		Saponin Cholesteride	
	Planaria	Snail	Planaria	Snail
0.9%	5 minutes	3.5 minutes	3–5 hours	1 hour
0.45%	4 minutes	4 minutes	10 hours	8 hours
0.09%	30 minutes	5 minutes	24 hours	20 hours
0.05%	60 minutes	6 minutes	40 hours	30 hours

"cholesteride," which is free of cholesterol particles, also kills the tadpoles in 5 hours and produces 50 per cent hemolysis in 20 hours. If we assume a very low content of free saponin, such as 1/200,000, which has the same hemolytic power as the above suspensions, we still cannot explain the killing action of these suspensions, because the tadpoles can tolerate this much saponin overnight.

Cholesterol Control

A suspension of cholesterol in water kills the tadpoles within 5 hours, possibly by the accumulation of crystals in their gills. If the suspension is filtered, it becomes harmless.

Cytolysis of Mammalian Tissues

Slices of liver, kidney and intestine of the rat were placed aseptically in 0.3 per cent Merck "cholesteride" and similarly in 0.3 per cent saponin for 7 hours in the ice box. Microscopic study of prepared sections revealed almost equivalent degree of cytolysis in both solutions. The liver was more damaged by saponin than by its "cholesteride," as judged by the dissolution of chromatin resulting in a cloudy homogeneous nucleus. The shrinkage of the liver tissue was enormous in both cases; many wide, long spaces were observed among the liver cords.

The intestinal epithelium was greatly dehydrated and damaged. The cells were almost devoid of cytoplasm. The nuclei were shriveled points. The effect of the "cholesteride" in this case was more striking than that of saponin.

The kidney nuclei were pycnotic in both solutions. The resistance of this organ was greater than that of the liver and the intestine.

Hemolytic tests were performed at the beginning and at the end of the immersion period of the tissue slices, and the "saponin cholesteride" was found nonhemolytic in 20 hours. It seems, then, that "saponin cholesteride" is as effective as saponin in causing the cytolysis of tissue cells.

Action of "Saponin Cholesteride" on Tadpole Development

It was mentioned under the properties of this compound that its toxicity as compared to saponin was greatly decreased, to such an extent that tadpoles could stand 0.9 per cent Baker "saponin cholesteride" for several hours with no serious damage. The tadpoles became quite irritable at first, moving rapidly in all directions, then they quieted down as if anaesthetized and remained so to the end of the period of immersion (1 to 2 hours). During this treatment their heart rate drops down to 30 beats a minute, as compared to 100 beats of the normal (chloretone anaesthetized) tadpoles. When washed and returned to water they remain "intoxicated" for an hour or so before they regain their normal activities and normal heart rate.

Tadpoles of various stages as well as cleaving eggs were treated by short immersions in "saponin cholesteride" and the effect on development was noted by comparing with the controls. All tadpoles were kept in finger bowls and fed boiled spinach leaves.

Trials with Cleavage Stages

Female frogs were induced to ovulate by injecting several pituitary glands in the abdomen of each. They were stripped into a suspension of minced testes. When all ova were in the two-cell stage, they were divided into four groups and placed in the following solutions:

1. 0.15 per cent Baker saponin
2. 0.9 per cent Baker "saponin cholesteride"
3. 0.0025 per cent KCN control
4. Water control

Within 5 hours, eggs in the four groups cleaved to the 16-cell condition. By 20 hours the eggs in the three agents reached the blastula or Stage 8,[3] while those in water reached late blastula or Stage 9. After 64 hours, both saponin and "saponin cholesteride" were still in blastula, and did not resume development after return to water. KCN treated eggs were in Stage 12 and water group in Stage 14. It was apparent that there was a generalized poisoning, due to continuous immersion in the solution, in the saponin and its "cholesteride."

Unhatched Batch (Stage 17)

These were immersed 2 hours daily in 0.9 per cent suspension for a period of two weeks. On the second day after treatment, 11 tadpoles hatched from the water control but none from the "cholesteride" group. On the ninth day, inspection of the organisms showed that the treated groups were smaller. They still had their suckers, whereas the controls had lost them. Treatment stopped after two weeks. On the fiftieth day from the beginning of the experiment, the treated animals averaged 2.8 cm. in length; their hind limbs were in Stage 7. The controls were 3.5 cm. and their limbs in Stage 11 (Fig. 3).

Hatched Tadpoles (Stage 19)

On the fifth day, it was noticed that the water control group were ahead of the "cholesteride" group in having their mouths open and their eyes more pigmented.

[3] In this paper, stage numbers are according to Rugh, 1948.

On the twenty-sixth day the tadpoles in water averaged 2 × 0.5 cm., while those in "cholesteride" were 1.4 × 0.3 cm.

Tadpoles of Stage 21

Daily immersion in 0.9 per cent "saponin cholesteride" for 15 days, after which they were returned to water. On the fiftieth day, the water controls had complete

FIGURE 3. A′, B′, C′ tadpoles showing retardation of growth by immersion in 0.9 per cent Baker "saponin cholesteride" for an average of one hour daily. A, B, C their untreated controls.

hind limbs, and beginnings of the fore limbs (Stage 19). The treated ones were around Stage 10. Among the controls there were some 4.5 cm. long and some 3.8 cm.; the treated were between 3.3 and 2.8 cm. (Fig. 3).

Tadpoles of Stage 23

In this series, the treated groups were immersed for 1.5 hours each in the same "cholesteride" as the previous cases, also for 15 days. On the eighth day, the controls had well formed jaws and four rows of teeth; the pigment covered only half the abdomen. The treated ones had one row of teeth, and much less abdominal pigmentation. Thin pieces of opercular fold were still visible. On the twentieth day the controls averaged 1.8 cm. × 0.45 against 1.2 × 0.35 cm. of the treated group (Fig. 3).

Summary

It is seen that there is a retardation in development of tadpoles due to "saponin cholesteride." Size as well as stage differences were noted. Microscopic examination of serial sections did not reveal any histologic difference in the treated animals. They were only smaller and retarded in the rate of development.

Action of Non-Hemolytic Saponin-Eosin

According to Noguchi (1906), eosin abolishes, at least partially, the hemolytic power of saponin if the mixture is exposed to the sun. The author kept a 0.3 per cent Merck saponin and 1.5 per cent eosin mixture in the sun for 65 hours. The excess eosin was adsorbed out by charcoal. Trials with this almost cleared solution showed that it was non-hemolytic and capable of retarding growth of onion roots as much as 75 per cent of the water control. Onion roots in similarly treated eosin control were retarded by 25 per cent only.

Discussion and Conclusion

The number of chemicals that can interrupt mitosis is enormous. Yet not all toxic substances do that. For example, the author tried several known poisons and found the following facts: one per cent Lysol kills onion roots but does not prevent mitoses completely. Bromine water diluted 9 times causes cytolysis and disruption of cells too drastic to show mitoses. One per cent pyridine was interesting: while cytoplasm, nuclei and nucleoli of the treated roots were in as perfect form as those of the best control roots, there were no mitotic figures at all. The roots, however, were dead. It warrants further study. Three-tenths of one per cent bile salt mixture kills and softens the roots in two days, yet there were some mitotic figures. It can be said that saponin is more than a general toxic substance, having a specific inhibition on mitosis in addition to its retardation of growth in a general way. Its non-hemolytic "cholesteride" does the same also but in higher concentrations. It is possible that this inhibitory action is the result of disturbances in the water content of the cells. There are several indications in support of this view: the shrinking and curvature of roots, actually becoming brittle, in the saponin and "cholesteride" solutions; the very hygroscopic nature of the compound; and the extensive shrinkage in animal tissues placed in saponin. The role of water in the

growth process is fundamental and a disturbance in water content might be the explanation of retardation in tadpole development and onion root growth. On the other hand, the compound might have an affinity to nuclear constituents. In fact the cytolysis of tissues in saponin, referred to previously (Butros, 1948), indicated a gradual disintegration of the nucleus as revealed by the Feulgen reaction. It is significant to note here that the "saponin cholesteride" had the same cytolytic power on mammalian tissues as saponin.

To conclude, it seems that the whole problem is worth further investigation. As most of this work was done on onion roots, more work should be turned to animal and tissue culture fields. Various other saponins might be tried and might give a milder toxic cholesteride with more inhibitory powers.

Summary

1. Onion root elongation was arrested within two days by 0.1 per cent Merck saponin and 0.3 per cent Baker saponin.

2. No mitotic figures were discerned in these cells; the nuclei of all cells were in the resting stage.

3. Lower concentrations, down to a certain point, reduced the rate of growth of the roots. Below that the very dilute saponins appeared to be growth stimulating.

4. A method of preparing a non-hemolytic suspension of saponin cholesteride is given.

5. The properties of non-hemolytic saponin cholesteride are described. Its toxicity to animals is much less than saponin; also it arrests onion root growth. It also retards the development of tadpoles. Its cytolytic power to mammalian tissues does not seem to have changed.

6. A non-hemolytic preparation of saponin-eosin is found to be partially inhibiting to onion root growth.

7. The possible explanations for this action are discussed.

LITERATURE CITED

Amerio, G. and Nora G. Dalla, 1942. Studies on experimental cytology: the influence of various chemical and physical agents on the structure of the plant cell. *Rend. Ist. Lombardo Sci., Classe Sci. Mat. Nat.*, **75**: 109–118.

Balansard, J. and F. Pellissier, 1943. Les saponines de Quillaya et Polygala agents de prolifération cellulaire. *C. R. Soc. Biol. Paris*, **137**: 523–525.

Balansard, J. and F. Pellissier, 1944. L'action des saponines sur la croissance. L'influence du traitement des semences sur les dernière phases de la végétation. *C. R. Soc. Biol. Paris*, **138**: 622–623.

Balansard, J. and F. Pellissier, 1945. Saponines et croissance chez *Solanum lycopersicum* L. *C. R. Soc. Biol. Paris*, **139**: 1098–1100.

Balansard, J., F. Pellissier and S. Conil, 1946. Action des saponines de Quillaya et de Sapindus sur le pouvoir absorbant, la germination et la croissance de *Zea mais* L. *C. R. Soc. Biol. Paris*, **140**: 140–142.

Butros, J., 1948. The effect of saponin on tissue and cancer cells *in vitro*. *Cancer Res.*, **8**: 221–230.

v. Euler, Hans, 1946. Influence of saponin and digitalis glycosides on seed germination and mitosis. Action of colchicine and x-rays. *Arkiv Kemi, Mineral. Geol.*, **A22** (10 pp.).

Frey, E., 1938. Einfluss der Saponine auf das Wachstum des Rattensarkoms. *Arch. Exper. Path. u. Pharmakol.*, **188**: 601–604.

Hideo, Kamon, 1928. The influence of different drugs on the growth of pulmonary and ventricular tissue of the hen embryo *in vitro*. *Folia Pharmacol. Japon.*, **7**: 86–114.

KOBERT-ROSTOCK, R., 1924. *In:* A. Heffter, Handbuch der experimentellen Pharmakologie. Bd. 2, S. 1476–1520.

MEYERSTEIN, W., 1910. Über die Beziehungen von Lipoidsubstanzen zur Hämolyse. *Archiv f. exper. Path. u. Pharmakol.*, **62**: 258–265.

NOGUCHI, H., 1906. The effect of eosin and erythrosin upon the hemolytic power of saponin. *J. Exp. Med.*, **8**: 268–270.

PONDER, E., 1948. Hemolysis and related phenomena. Grune and Stratton, New York. Pp. 178–182.

RUGH, R., 1948. Experimental embryology. Burgess Publishing Co., Minneapolis.

WENT, F. W. AND K. V. THIMANN, 1937. Phytohormones. The Macmillan Co., New York.

WINDAUS, A., 1909. Über die Entgiftung der Saponine durch Cholesterin. *Ber. Deutsch Chem. Ges.*, **42**: 238–246.

STROKE AMPLITUDE AS A FUNCTION OF AIR DENSITY IN THE FLIGHT OF DROSOPHILA

LEIGH E. CHADWICK

Medical Division, Army Chemical Corps, Army Chemical Center, Maryland

With the accumulation of the necessary quantitative information, it is gradually becoming apparent that the complex wing movement of insects may represent the integration of several components, so interrelated that variation in any one is compensated precisely by appropriate changes in the others. Thus, for example, strong evidence in support of the theory that wingbeat frequency in the higher Diptera is regulated by the loading of the flight muscles has been put forward by Pringle (1949); in the case of Drosophila the relationship between frequency and atmospheric density agrees qualitatively with this point of view, emphasizing the fact that a major element of the load is the air resistance which the wings must overcome (Chadwick and Williams, 1949). Here, however, the observed changes in frequency were insufficient to compensate completely for the variation in load imposed by density change, indicating clearly that elements of the wing motion other than the frequency must also vary in an orderly manner if equilibrium is to be preserved.

From studies with vertebrate muscle (see Fenn, 1945), as well as from the few pertinent investigations with insects (Solf, 1931; Kraemer, 1932; Cremer, 1934), it is well known that not only the speed but also the extent of muscular shortening may be reduced by an increase in load. Regular changes in amplitude resulting from alteration in atmospheric density are therefore wholly to be expected. The problem in the present study has been the investigation of such changes from the viewpoint of their contribution to the integration of the flight mechanism as a whole.

One may reason, *a priori*, that in the analysis of insect flight the stroke amplitude should be as important as the frequency with which the wings are moved, since in theory the work done per beat is proportional approximately to the square of each of these factors. Evidence supporting this deduction has been produced, as far as frequency is concerned, by measuring respiration and rate of wingbeat concurrently during flights in which the level of activity was altered progressively by fatigue (Chadwick and Gilmour, 1940; Chadwick, 1947). However, such data suggest also that, unlike frequency, amplitude does not vary systematically under these experimental conditions, an inference which Williams (1941) has confirmed photographically.

It is well known, however, that in other circumstances amplitude may change considerably. Differences in the amplitude of right and left wings were observed by Voss (1914) and Stellwaag (1916), who discovered in this asymmetry the mechanism for steering in the horizontal plane. Moreover, Hollick (1940) has measured changes in the torque about the transverse axis which were dependent upon variation in the amplitude of both wings simultaneously, so that steering in the vertical plane also appears to be achieved through control of amplitude.

That variations in amplitude are not related solely to steering is attested by various other observations. Thus, Hollick (1940), like Magnan (1934), has described an inverse relationship between amplitude and the airspeed of the insect; while Sotavalta (1947) noted that smaller amplitudes are characteristic of insects flying freely in nature than of the same species when fastened, and that amplitude often increases when an insect carries a heavier load, as may be observed in predatory species with prey or in mosquitoes after a blood meal. Some of these facts may seem at first in contradiction with the decrease of amplitude noticed in fastened Drosophila at air densities above normal (Chadwick and Williams, 1949), but probably there is no real disagreement. If one makes the likely assumption that a fastened insect is already bearing a full load, any further burden on the muscles, as from an increase in the resistance of the air to the passage of the wings, can only result in a decrease in wingbeat frequency, amplitude, or both. A free-flying insect, on the other hand, ordinarily has some fraction of power in reserve. That the stroke amplitude of fastened Drosophila does in fact change systematically with alterations in air density is demonstrated by the observations described below.

Method

The experimental method was essentially that used previously by Hollick (1940) and Williams (1941), in which the envelope of the wing motion is recorded photographically by making a time exposure of a fastened insect in flight.

Females of *D. virilis* Sturtevant were anesthetized with carbon dioxide gas, and each specimen fastened to a wire by means of paraffin. The mount was then adjusted in a cylindrical holder which carried a retractable spring platform with which the feet of the fly were placed in contact, and the holder was slipped into the flight chamber, as shown in Figure 1. Care was taken to orient the insect so that flight was in a horizontal plane, with the specimen either head-on or sideways to the camera. Flight was induced by bringing a small permanent magnet, outside the chamber, into position beneath the platform.

The chamber consisted of a glass cylinder of heavy combustion tubing, 6 inches long and one inch in internal diameter, enclosed in a frame cut from stainless steel pipe. Threaded steel fittings provided for closure at both ends. As illustrated in Figure 1, one fitting carried inlet and outlet tubes for exchanging gas within the chamber; the other served to clamp a lucite flat, 0.25 inch thick, against the end which faced the camera.

Illumination was supplied by microscope lamps, one at either side of the chamber; these were turned on only while a record was being made. Exposure times of 1 to 2 seconds, aperture of f/11, gave head-on photographs at about 1 diameter on Super-XX film from which measurements of vertical amplitude could be made satisfactorily (Fig. 2). With the insect in side view, better pictures were obtained with an exposure of 0.2–0.5 second at aperture f/16 (Fig. 3).

Pressure within the chamber was varied by adding oxygen from a commercial cylinder, or by means of a vacuum pump. Positive pressures were read from a gauge, negative pressures from a mercury manometer against which the gauge had been calibrated up to a pressure of 3 atmospheres. Higher pressures were measured by gauge alone, assuming a linear extrapolation of the calibration data. The

atmosphere within the chamber was kept moist by evaporation from a piece of dampened filter paper. After each change of pressure a few minutes were allowed for equalization of the chamber with the laboratory temperature of 27 ± 1 degrees C.

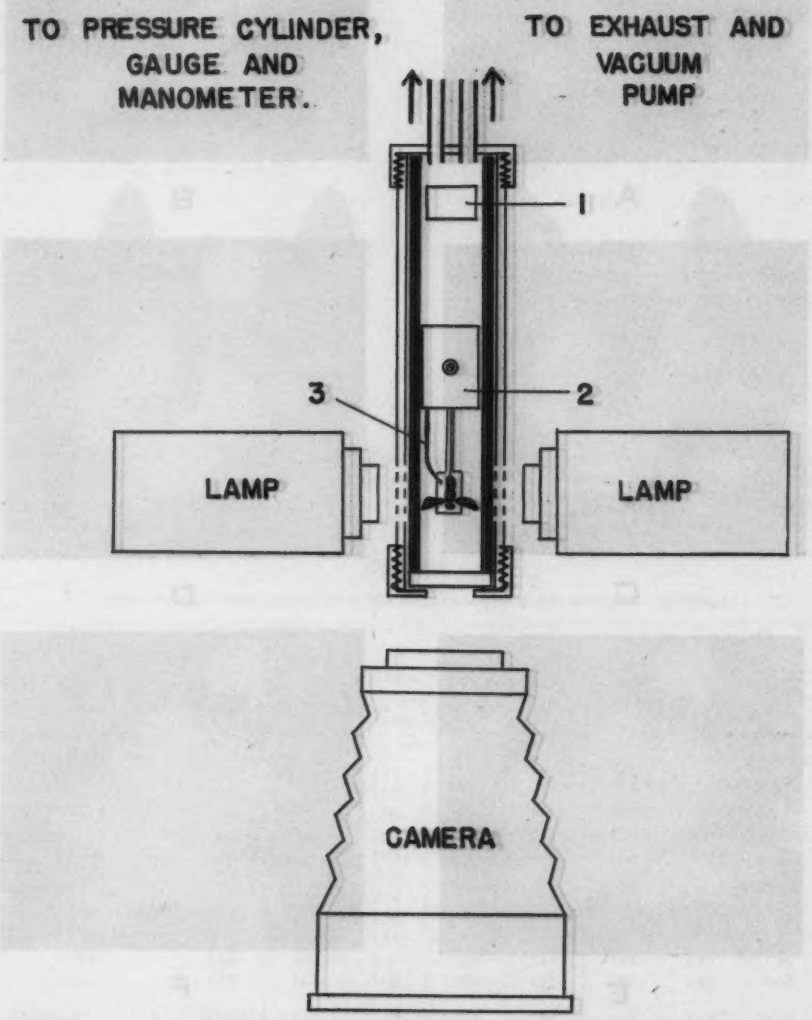


FIGURE 1. Diagram of apparatus. 1, piece of moistened filter paper; 2, holder for insect mounting and retractable platform; 3, stop for platform. For a description of other details, see text.

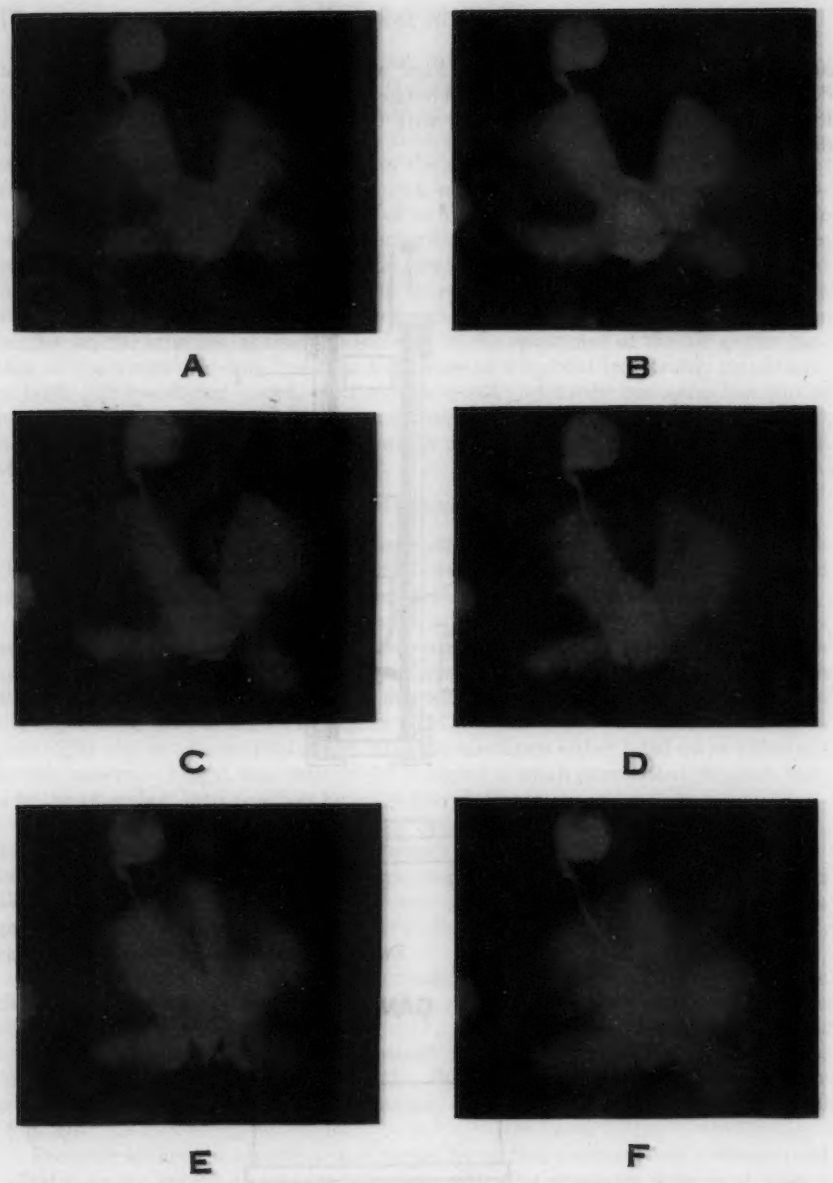


FIGURE 2. Representative series of head-on photographs to show alteration in stroke amplitude of a specimen of *D. virilis* in successive flights at different air pressures, as follows: a. 3863 mm. Hg; b. 2311 mm. Hg; c. 1520 mm. Hg; d. 760 mm. Hg; e. 380 mm. Hg; f. 190 mm. Hg. Records obtained at 3087 mm. Hg and at 95 mm. Hg have been omitted.

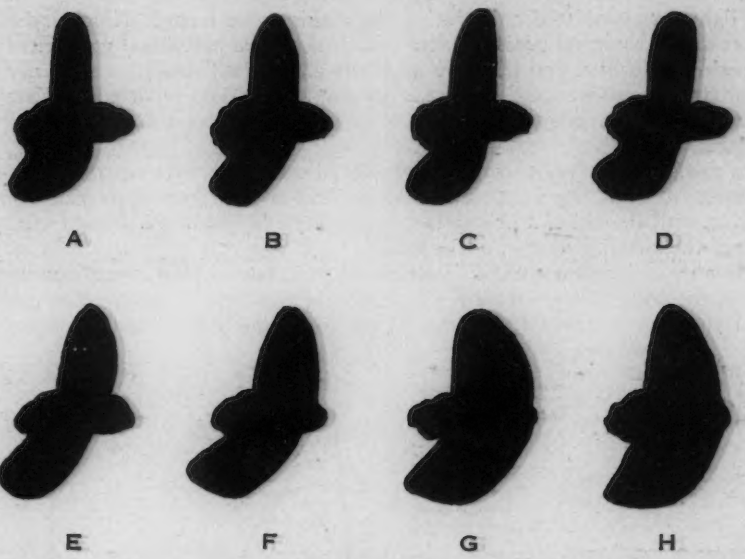


FIGURE 3. Series of silhouettes, traced from enlargements of original photographic records, to show changes in envelope of wing movement of a specimen of *D. virilis* during successive flights at different air pressures. Left lateral view. a. 3863 mm. Hg; b. 3087 mm. Hg; c. 2311 mm. Hg; d. 1520 mm. Hg; e. 760 mm. Hg; f. 380 mm. Hg; g. 190 mm. Hg; h. 95 mm. Hg.

TABLE I

D. virilis: vertical amplitude of wingbeat as a function of air pressure and density

Pressure in mm. Hg	3863	3087	2311	1520	760	380	190	95
Density in gm. per L.	5.97	4.76	3.56	2.34	1.16	0.57	0.28	0.13
Specimen Number	Stroke Amplitude in Degrees of Arc							
516–3	102	103	112	112	127	129	—	137
517–1	100	103	109	98	117	138	138	138
517–2	100	92	96	103	111	127	149	143
517–3	80	99	105	90	111	106	121	138
518–2	104	113	105	102	118	127	124	121
518–3	110	111	113	128	123	139	134	141
518–4	102	114	114	119	124	126	132	134
518–5	99	114	120	130	131	144	147	129
519–1	94	113	116	121	148	148	152	158
519–2	114	124	117	127	135	154	137	139
619–2	119	121	108	106	124	132	140	142
Average	103	110	110	113	124	133	138	138
Standard Error	±3.1	±2.9	±2.1	±4.1	±3.3	±3.9	±3.2	±2.8
Number of Observations	11	11	11	11	11	11	10	11

Flight was permitted only during the making of a record. Each insect was first studied at normal pressure, after which the same individual was tested over the series of positive and negative pressures shown in Table I. Ordinarily only one or two records were taken at each pressure. The data reported in the several tables represent average figures in all cases where more than one record was available.

In analyzing the photographs, each record was projected and traced at about 8 diameters. The apparent amplitude, in head-on view, was then read from the drawing with the aid of a protractor, as the angle between the two extreme positions of the anterior margin of the wing.

Because of foreshortening in pictures taken in lateral view, the apparent position of the wing in such records may differ appreciably from the true position. The latter was therefore determined by reconstruction before measurements were made, as follows (see Fig. 4):

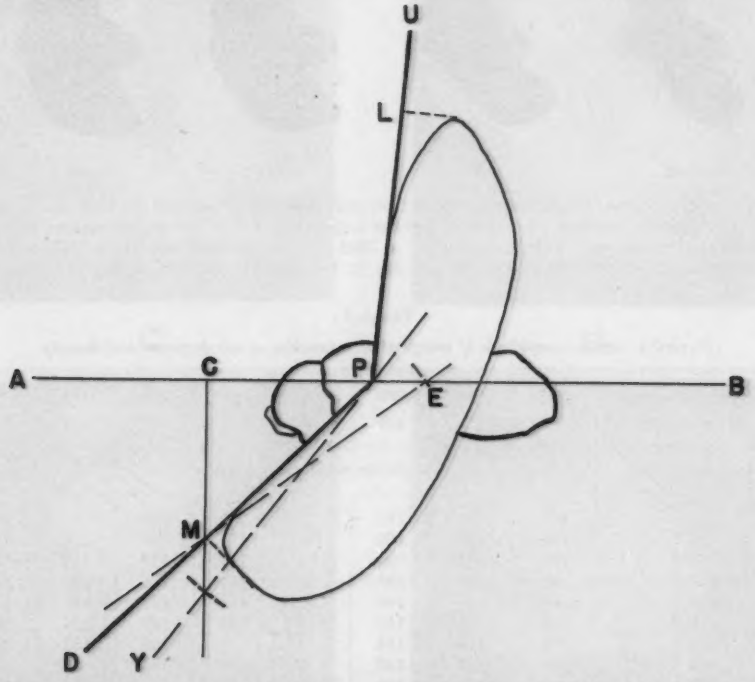


FIGURE 4. Diagram to show method of reconstructing from a photograph in side view the true angular displacement of the wing during the downstroke. For explanation, see text.

A horizontal line, AB, was drawn through the wing articulation, P, on the tracing; and extensions, UP and DP, of the costal margin in the extreme up and down positions were constructed. The true relative length of the wing, PL, was determined by dropping a perpendicular from the wingtip to UP in a record

of the same specimen taken at normal pressure, where it was known from head-on photographs that the costa is approximately in a vertical, parasagittal plane. The apparent length of the wing in the downstroke, PM, was similarly laid off; and a perpendicular, CM, drawn from M to AB. The true position of the costa in the downstroke was then found as the line connecting (1) P, and (2) the intersection of line CM with an arc of length PL centered at P. This reconstructed position of the costa is represented by the broken line, PY, in the figure.

Angles UPA and YPA were now recorded.

A similar reconstruction could be made for the position of the costa in the upstroke, at pressures other than normal, or in the downstroke in head-on view, but such corrections proved insignificant in practice and were therefore omitted.

TABLE II

D. virilis: variation in components of stroke amplitude as a function of air pressure and density

Pressure in mm. Hg	3863	3087	2311	1520	760	380	190	95	
Density in gm. per L.	5.97	4.76	3.56	2.34	1.16	0.57	0.28	0.13	
	Displacement Relative to Frontal Plane in Degrees of Arc								Type of Record
Upstroke	72	77	76	79	86	93	94	93	Front view
Downstroke	−31	−33	−34	−34	−38	−40	−44	−45	Front view
Downstroke	−34	−38	−35	−34	−38	−39	−47	−43	Side view
	Displacement Relative to Transverse Plane in Degrees of Arc*								
Upstroke	−5	−1	−4	−3	−7	−6	−5	−5	Side view
Downstroke	20	20	29	22	29	28	25	27	Side view

Each datum is the mean of from 6 to 30 measurements.

* Negative values here indicate displacement in a posterior direction.

Angle YPA gives a measure of the true forward displacement of the costa at the conclusion of the downstroke. The vertical displacement may also be obtained from the same record by drawing the line through M and the intersection, E, with the horizontal, AB, of an arc of length PL centered at M. The angle MEP is then a measure of the actual depression of the costa below the horizontal. Average values for angle MEP were obtained in this way from records of 6 animals which flew at all pressures. As indicated in Table II, such calculated values are in substantial agreement with the mean values measured directly from head-on pictures of other flies.

RESULTS

Table I records the total vertical amplitudes measured from head-on records of 11 specimens which, with one exception, flew at all 8 pressures tested. From the same records, the displacement of the costa above and below the horizontal was determined separately; the averages of these values are shown in Table II, which includes for comparison the average downward displacements obtained

from 6 sets of records in side view (see the preceding section for the method of measurement). Also shown in Table II is the average angular displacement of the costa in the fore and aft direction at the conclusion of up- and downstroke, as determined from photographs taken in side view. Other alterations in the envelope of the wing movement in response to density change are illustrated by the series of silhouettes in Figure 3.

Discussion

The data depict a significant trend toward greater amplitudes in the transverse plane at lower densities, and vice versa (Table I). When density is increased, both the upward and downward excursions of the wing are curtailed, while there is little change in the extreme fore and aft displacement of the wing (Table II). However, the trajectory of the wing varies in such a way as to give a broader envelope at lower densities when recorded in lateral view (Fig. 3).

The relationship between vertical displacement of the wing and density is linear on a double logarithmic scale, permitting calculation of the following regressions:

(a) limit of displacement above body axis (a_u) on density (ρ),

$$\log a_u = 1.9140 - 0.0885 (\log \rho - 0.2344); \quad (1)$$

(b) limit of displacement below body axis (a_d) on density (ρ),

$$\log a_d = 1.5688 - 0.0976 (\log \rho - 0.0944); \quad (2)$$

(c) total vertical amplitude (a) on density (ρ),

$$\log a = 2.0721 - 0.0934 (\log \rho - 0.2344). \quad (3)$$

In the calculation of equations (1) and (3), the empirical points at the lowest density, 0.13 gram per liter, were omitted, since they deviate significantly from the lines defined by the other observations. This discrepancy may be due to the fact that the upward displacement of the wing has already reached its physical limit, not greatly in excess of 90 degrees, at densities greater than 0.13 gram per liter. Another possibility is that performance at the lowest density was influenced to some extent by oxygen lack. In this series of experiments no attempt was made, as was done in the case of frequency (Chadwick and Williams, 1949), to rule out variation in oxygen tension and in total pressure as such as factors in the results obtained, since with the possible exception just noted, one sees no reason to suppose that either is implicated in the amplitude changes observed. But, in moist air at 27 degrees C. and at a density of 0.13 gram per liter the total pressure is 95 mm. Hg and the partial pressure of oxygen only some 15 mm., a value known to be close to the limit consistent with brief interrupted flight. It was frequently difficult to obtain a satisfactory response from the specimens at this pressure, and it is possible that those which flew were in a subnormal state. The remaining observations show but little scatter about the lines calculated, as judged by the chi-square test for dispersion. One may conclude then that amplitude is proportional, on the average, to the -0.09 power of density.

This finding may now be examined along the lines followed in the previous study of the dependence of wingbeat frequency on gas density.

$$P_o \propto mv^2f; \tag{4}$$

where P_o equals power output; m is the mass of air moved per stroke; v, the average velocity imparted to mass m; and f, wingbeat frequency. Or

$$P_o \propto V_s\rho v^2f, \tag{4a}$$

since m equals $V_s\rho$, where V_s is stroke volume and ρ, air density.

Now, it is apparent that amplitude is a factor in both the stroke volume, V_s, and the velocity of the wing, v_w. To the latter the air velocity, v, must, in turn, be proportional. Consider that an approximation of the stroke volume, in terms of the wing dimensions, is given by regarding the volume swept out by the wing as the segment of a cylinder with radius equal to the wing length, L, and with height equal to the product of mean wing width, W, and the sine of the angle of attack, α. Then

$$V_s \text{ (both wings)} = 2\pi L^2W \sin \alpha(2a/360°), \tag{5}$$

where a is the stroke amplitude. For a similar calculation of the mean velocity imparted to the air, it is necessary to take account of the shape of the wing, which is narrowest at the base. The mean velocity is therefore that produced by a unit area situated somewhat further than half the wing length from the articulation. Calling this radius L_m, we may then write

$$v \propto v_w \propto 2\pi L_m(2a/360°)f. \tag{6}$$

On substitution of expressions (5) and (6) for stroke volume and velocity, respectively, into equation (4a) above, one obtains:

$$P_o \propto a^2f^2\rho \sin \alpha. \tag{7}$$

Reference to the study by Chadwick and Williams (1949) shows that wingbeat frequency (f), for different individuals, varies on the average as the -0.09 power of density; that is, in the same quantitative manner as amplitude. By substituting $\rho^{-0.09}$ for both amplitude (a) and frequency (f) in equation (7), one finds

$$P_o \propto \rho^{0.46} \sin \alpha. \tag{8}$$

Provided then that no further factor of importance has been overlooked, one may judge that equilibrium can be maintained under constant power output only by variation of sin α as $\rho^{-0.46}$.

Reasons for supposing P_o constant, which were discussed fully in the previous publication, may be summarized as follows:

(a) P_o is equal to the product of power input, P_i, and an efficiency factor, e;

(b) P_i, measured in terms of oxygen consumption at different densities, is essentially constant;

(c) any factor which is to balance the relationship between frequency, amplitude, and density must show *continuous* exponential variation over the full range of densities investigated;

(d) a large increase in efficiency at higher densities, required if the relationship is to be balanced by alteration in P_o while P_i remains constant, is unlikely.

Although one is led by this reasoning to reject changes in efficiency as a plausible solution of the problem, it has been pointed out by O. Sotavalta (1947, 1951) that this treatment neglects energy losses due to inertia of the wing. These, he feels, may be considerable, inasmuch as the wing is reversing its direction of movement twice per cycle at frequencies of the order of 200 per second. Since there is appreciable variation in both frequency and amplitude, and hence, by inference, in rotational inertia of the wing, when density is altered, one must admit that the assumption of constant power output will be in error to the extent that efficiency is changed by variation in this loss. The possible significance of this factor in the flight of *D. virilis* may be assessed as follows:

$$E = 1/2\ I(\omega)^2; \tag{9}$$

where E is the mean kinetic energy of rotation of the wing at any instant; I, the rotational inertia of the wing; and ω, its mean angular velocity, *i.e.*,

$$\omega = 2\pi(2a/360°)f. \tag{10}$$

Now,

$$I = m_w r^2, \tag{11}$$

where m_w is the mass of the wing and r the radius of gyration, which may be taken as approximating $\sqrt{1/L \int_0^L r^2 \cdot dr}$, or $L \times 3^{-0.5}$, L being the wing length.

If E is dissipated twice per stroke, the power loss per wing is $2fE$; per insect,

$$P_e = 4fE. \tag{12}$$

Combining equations (9), (10), (11) and (12) gives the solution (on substitution of the measured average values, for *D. virilis*, of 2×10^{-1} cm. for wing length, L, and 3.625×10^{-6} gram for wing weight, m_w):

$$P_e = 1.1784 \times 10^{-10}\ \text{gm.cm.}^2 \times f^2 \times a^2. \tag{13}$$

From this equation, the values of P_e at different densities have been worked out, using our measurements of average f and a, and are given in Table III.

TABLE III

D. virilis: variation at different air densities of power loss due to inertia of wings

Density ρ grams per liter	Frequency* f cycles per second	Amplitude* a degrees of arc	Power Loss P_e ergs per second	Power Available for Useful Work P_0 ergs per second	$P_e/P_0 \times 100$ per cent
5.97	205	105	11.19	204.49	5.5
4.76	210	107	12.49	203.19	6.1
3.56	215	110	14.17	201.51	7.0
2.34	223	115	17.28	198.40	8.7
1.16 (1 atm.)	238	123	24.04	191.64	12.5
0.57	254	131	33.14	182.54	18.2
0.28	271	140	45.97	169.71	27.1
0.13	290	150	64.67	151.01	42.8

* Frequencies computed from data of Chadwick and Williams (1949); amplitudes, from equation (3) above; for full explanation of other calculations see text.

A figure for the *effective* power output at normal density, 25 degrees C., is available from unpublished data of Williams on *D. repleta*; the maximal value measured was 159.74 ergs per second. Discounting the species difference, since *D. virilis* and *D. repleta* have approximately the same size and flight characteristics, and allowing for a measured frequency increase of 421 beats per minute per degree C. (since $P_o \propto f^3$), we can estimate the effective power output at normal density under the conditions of our present experiments at 191.64 ergs per second. If wing inertia, amounting to 24.04 ergs per second, is a complete loss, this would represent a total output of 215.68 ergs per second. The fraction available for moving air at each density may now be calculated by subtracting from 215.68 ergs per second the corresponding calculated loss, P_e, due to inertia. Thus we obtain the values shown in column 5 of Table III. Two significant facts which emerge from these calculations are: (1) the power loss due to wing inertia in the flight of Drosophila probably does not exceed 45 per cent of the effective power output even under the most adverse conditions, and is normally much smaller than this; (2) although this loss varies in the proper manner, increasing at low densities and vice versa, and in proportion to $\rho^{-0.45}$, it is not of sufficient magnitude to provide balance in the relationship of equation (8) in the absence of variation in sin α.

A few additional comments are needed in reference to these calculations of the wing inertia. The values derived above may be somewhat low, since the wing trajectory includes a varying horizontal component (see Fig. 3) not contained in our formula for the angular velocity. Another inaccuracy stems from the fact that the true radius of gyration is, of course, unknown, though one doubts that it is greater than the length assigned, since the wing, though narrower, is appreciably more massive toward the base. Granting that possibly somewhat higher estimates should be given for P_e than those shown in Table III, this error is counterbalanced to some extent by the probability that the value cited for the effective power output is low. The figure quoted is merely the maximal output determined under a particular set of experimental conditions, and there is reason to believe that somewhat greater outputs might be measured with improved techniques. Finally, the assumption that the kinetic energy of the wing becomes a total loss, twice per cycle, is open to question. A portion of this energy must be expended against the air and contribute useful work. Although the larger fraction in each half cycle is dissipated, no doubt, in stretching the antagonistic sets of muscles, one must reckon also with the possibility, given the very short duration of the cycle, that a proportion of even this fraction is reabsorbed (cf. Hill, 1950). Some energy may also be recaptured through elastic deformation of the thoracic cage. Weighing these various considerations together, it seems improbable that the actual loss of power due to wing inertia amounts to a fraction of the power output significantly greater than indicated in Table III.

We are thus left with a choice of assumptions: either the overall efficiency of flight varies, in some manner as yet unexplained, about as the -0.4 power of density, or the angle of attack of the wings on the air changes in the same proportion. Believing that most animals are fairly well adjusted to their normal environment, one is reluctant to postulate increases of efficiency amounting to something over 100 per cent when Drosophila are placed in the wholly unnatural situation of being forced to fly at an air pressure of 5 atmospheres. For this

reason the alternative, a comparable increase in the angle of attack at 1/8 atmosphere and an equivalent reduction at 5 atmospheres, seems the more acceptable, although we regret our inability to settle the question experimentally. If, as suggested by the silhouettes of Figure 3, the quantity $V_s\rho v^2$ varies somewhat more rapidly with changing density than has been estimated on the basis of our measurements of frequency and vertical amplitude, the actual rate of variation required in the angle of attack may be even less than we have been led to postulate above. It is to be hoped that methods for attack upon the several unknowns still remaining in this problem will be developed.

The writer is indebted to Dr. C. M. Williams for valuable discussion in regard to all phases of this investigation.

Summary

The amplitude of wingbeat of *Drosophila virilis* Sturtevant in moist air at a temperature of 27 ± 1 degrees C. and at a series of gas pressures ranging from 3863 mm. Hg to 95 mm. Hg, was recorded photographically.

The vertical amplitude of the wingbeat decreased progressively with increase in air pressure. The excursion of the wing was curtailed in the same proportion at both extremes of the stroke. These changes are attributed to alterations in atmospheric density which resulted from the change in gas pressure. It is calculated that amplitude varies approximately as $\rho^{-0.09}$.

No significant change was observed in the limits of angular excursion of the wing in a fore and aft direction, although the envelope of the wing movement, as seen in lateral view, broadened as pressure was reduced.

It is shown that the measured changes in amplitude and frequency compensate only in part for the change in load which results from alterations in air density. The possible role of power losses due to wing inertia is discussed, and it is concluded that these represent only a minor factor in the observations. Arguments are presented which suggest that alterations in gas density provoke compensatory changes not only in wingbeat frequency and stroke amplitude, but also in the angle at which the wings attack the oncoming stream of air. The degree of compensation required would be provided by doubling the angle of attack at 95 mm. Hg and halving it at 3863 mm. Hg.

LITERATURE CITED

Chadwick, L. E., 1947. The respiratory quotient of Drosophila in flight. *Biol. Bull.*, **93**: 229–239.

Chadwick, L. E. and D. Gilmour, 1940. Respiration during flight in *Drosophila repleta* Wollaston: the oxygen consumption considered in relation to the wing-rate. *Physiol. Zool.*, **13**: 398–410.

Chadwick, L. E. and C. M. Williams, 1949. The effects of atmospheric pressure and composition on the flight of Drosophila. *Biol. Bull.*, **97**: 115–137.

Cremer, E., 1934. Anatomische, reizphysiologische und histologische Untersuchungen an der imaginalen und larvalen Flugmuskulatur der Odonaten. *Zool. Jahrb., Abt. Physiol.*, **54**: 191–223.

Fenn, W. O., 1945. Contractility. *In* Höber, R., Physical Chemistry of Cells and Tissues. The Blakiston Company, Philadelphia.

Hill, A. V., 1950. The thermodynamics of the active state of muscle. *Biochem. J.*, **46**: xli.

HOLLICK, F. S. J., 1940. The flight of the dipterous fly, *Muscina stabulans* Fallén. *Philos. Trans. Roy. Soc. London, Ser. B*, **230**: 357–390.

KRAEMER, F. K., 1932. Der Einfluss der Temperatur auf den Zuckungsverlauf von *Dytiscus marginalis* und *Lucanus cervus*. *Zool. Jahrb., Abt. Physiol.*, **52**: 86–117.

MAGNAN, A., 1934. La locomotion chez les animaux. I. Le vol des insectes. Hermann et Cie., Paris.

PRINGLE, J. W. S., 1949. The excitation and contraction of the flight muscles of insects. *J. Physiol.*, **108**: 226–232.

SOLF, V., 1931. Reizphysiologische Untersuchungen an Orthopterenmuskulatur. *Zool. Jahrb., Abt. Physiol.*, **50**: 175–264.

SOTAVALTA, O., 1947. The flight-tone (wing-stroke frequency) of insects. *Acta Ent. Fennica*, **4**: 1–117.

SOTAVALTA, O., 1951. The effect on the wing-stroke frequency in some insects, caused by the mutilation and loading of the wings and by subatmospheric air pressures. Cambridge University Thesis.

STELLWAAG, F., 1916. Wie steuern die Insekten während des Fluges? *Biol. Zentralbl.*, **36**: 30–44.

VOSS, F., 1914. Vergleichende Untersuchungen über die Flugwerkzeuge der Insekten. *Verh. d. deutsch. zool. Gesell.*, **24**: 59–90.

WILLIAMS, C. M., 1941. A morphological and physiological analysis of the flight of Drosophila, with special reference to the factors controlling the frequency of wing beat. Harvard University Ph.D. Thesis.

THE SOURCES AND ACTIVITIES OF TWO CHROMATOPHOROTROPIC HORMONES IN CRABS OF THE GENUS SESARMA. I. EXPERIMENTAL ANALYSES[1]

MASASHI ENAMI

College of Fisheries and Animal Husbandry, Hiroshima University, Fukuyama, Japan

As is well known (Brown, 1944; Hanström, 1947, etc.), the significance of the sinus gland as the principal source of chromatophorotropic hormone or hormones has been well substantiated by investigations, done with a large number of species in a taxonomically wide range of crustaceans since the first description of this incretory tissue by Hanström (1933). On the other hand there is a growing knowledge of the possible presence of certain hormone sources outside the sinus gland which is owed chiefly to recent continued efforts by Brown and his associates (1940–1947). They have demonstrated hormone production within the central nervous organs first in Crago, and are extending the research to other kinds of crustaceans. Thus, in the light of present information, we may infer that chromatophorotropic hormones that are directly concerned with the physiological coordination of pigmentary effector systems of crustaceans are roughly classified into two broad categories, *viz.*, the one originating in the sinus gland and the other in the nervous tissues.

Nevertheless, we are as yet only fragmentarily informed of details in connection with the actual number of hormones belonging to these respective categories, the essential activity of each of the hormones, the mode of interaction among them, etc., which is still keeping us from postulating certain generalizations with respect to the endocrine mechanism for the control of crustacean chromatophores.

Having been engaged in physiological as well as histological analyses of endocrine organizations in crabs of the genus Sesarma, with special reference to the production of chromatophorotropic hormones, I obtained some results which may furnish information concerning some of the pending problems just mentioned.

Materials and Methods

The animals used in the physiological analysis of the endocrine control of chromatophores were three representatives of the genus Sesarma, *viz.*, *S. intermedia*, *S. haematocheir* and *S. dehaani*, the most familiar brackish-water crabs in Japan. Adults of the respective species weighing ca. 30 g. (males) or ca. 25 g. (females) collected in the field were kept alive in the laboratory until required for experimentation.

For the assay of chromatophorotropic hormones present in the crabs, tissues and organs were dissected out from individuals showing no sign of ecdysis. In

[1] The present work was supported by a grant-in-aid from the Ministry of Education of Japan during the fiscal years of 1947 and 1948. Research facilities were provided by the Mitsui Institute of Marine Biology, Shimoda, Japan, where the work was carried out.

order to reject specimens in the molting phase, the early indication of the phase at the epipodite of the first maxilliped (cf. Drach, 1939) was resorted to. Isolated tissues and organs were separately ground with quartz sand to be extracted twice with distilled water. The insoluble fraction was centrifuged off, and the supernatant fluid was sealed in a glass tube and dipped into boiling water for 10 minutes. Such a preparation could be preserved for weeks and months without significant loss of hormone activity. When needed for the assay, the samples were evaporated *in vacuo* to be re-extracted with physiological saline. The final extracts were freed by filtration from coagula formed in the process of previous heating. Concentrations of extracts were expressed in terms of the number or weight of the respective tissues or organs theoretically contained in the administered doses, which were designated as T.E. (tissue equivalents). The chromatophorotropic activity of the extracts was tested chiefly upon the chromatophores of juvenile forms of *Sesarma haemato-*

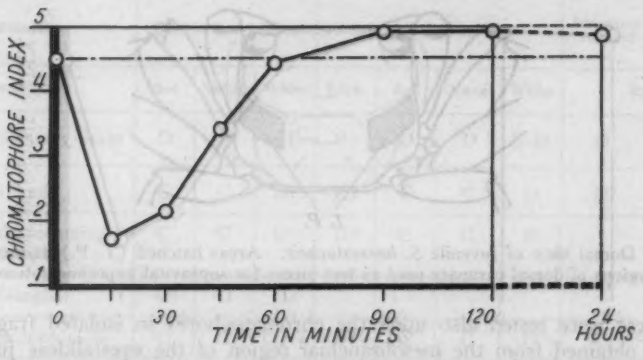


FIGURE 1. Curve showing time relation of pigmentary response of black chromatophores of juvenile *S. haematocheir* following amputation of both eyestalks.

cheir with carapace dimensions less than 20 mm. × 18 mm. Such individuals, in spite of the marked sluggishness in the chromatic display of adult forms of the three species of Sesarma, show a high chromatic activity, changing coloration from creamy yellow to chestnut brown uniformly over the whole dorsal aspect of the body including the walking legs. Background responses are lacking like in other color-changing crabs, and, under laboratory conditions during the day, the immature crabs exhibit darkening that is considerably subject to fluctuations of environmental factors such as the intensity of light, moisture and heat.

Upon bilateral amputation of the eyestalks, remarkable blanching of short duration takes place, which is followed by a process of re-darkening, and the eyestalkless animal eventually attains maximum darkness of body shade that is regarded to be the equilibrium state of pigmentary activity in the day, characteristic of the eyestalkless condition. Mainly responsible for such macroscopic changes of coloration are the brownish black chromatophores (Fig. 1), but the animal is provided with additional chromatophores, *viz.*, the red, the vermilion and the white cells, which, though limited in the chromatic effects, are also significant for the pigmentary behavior.

In the equilibrium state of the pigmentary activity of the eyestalkless animal, the black chromatophores are seen in a state of maximum pigment dispersion, the red and the vermilion cells together are in states of approximately semi-dispersion, and the white ones are in the state of nearly maximum concentration. Such states of the chromatophores were used *in vivo* and *in vitro* as test objects for the assay of the chromatophorotropic effectiveness of the extracts prepared from adult Sesarma.

Tissue or organ extracts re-extracted with known amounts of van Harreveld's solution buffered to pH 7.5 (mean pH value of the body fluid of Sesarma in the intermolt phase) were administered to the test animals that had been eyestalkless for at least two days, by injection into the thoracic musculature through the arthrodial membrane at the basal articulation of the second walking leg. Five one-hundredths cc. was injected per individual at a time; a lot of 15 test animals was employed for one assay.

FIGURE 2. Dorsal view of juvenile *S. haematocheir*. Areas hatched (T. P.) at mesobranchial regions of dorsal carapace used as test pieces for supravital experimentation.

Extracts were tested also upon the chromatophores in isolated fragments of carapace, obtained from the mesobranchial region of the eyestalkless juvenile *S. haematocheir* (Fig. 2). As was already remarked by Brown and Meglitsch (1940), chromatophores surviving in isolated carapace fragments usually show a considerably lowered reactivity, and significant pigmentary displays are often brought about by only those distributed at the periphery of each fragment. In the present experiments, however, experience taught that frequently repeated rinsing with van Harre-

TABLE I

Size and density of four types of chromatophores in mesobranchial region of juvenile S. haematocheir of carapace dimensions of 16 mm. × 14 mm. Measurements upon eight individuals

Size and Density \ Chromatophore	Black	Red	Vermilion	White
Optical Diameter at Maximum Pigment Concentration	33μ–55μ commonly ca. 38μ	11μ–33μ commonly ca. 22μ	11μ–22μ commonly ca. 17μ	15μ–33μ commonly ca. 22μ
Number in 1 mm.² of Carapace Area	87–165	6–28	74–174	96–252
Mean ± σ	120.4 ± 21.1	18.6 ± 6.9	117.9 ± 29.7	166.0 ± 54.8

veld's solution of carapace fragments directly following removal from the body was fairly good for maintaining the reactivity of chromatophores, so that use was made of carapace fragments washed for one hour in the solution that was renewed every 15 minutes. Such a treatment did not seriously affect the initial pigmentary conditions of chromatophores in the eyestalkless animal; responsiveness of the latter was preserved for an additional 10 hours under 25°C. In the assay of the extracts, 10 pieces of carapace fragments of 1 mm. × 2 mm. obtained from 10 different eyestalkless individuals were immersed in 3 cc. of the respective extract. The useful idea of T.E. was adopted too for the expression of concentrations of extracts.

TABLE II

Table summarizing chromatophorotropic activities of extracts from adult Sesarma as tested by injection upon Paratya, Ocypoda and Megaligia

	Test Animal	*Paratya* Eyestalkless			*Ocypoda* Eyestalkless				*Megaligia* "Blinded"	*Megaligia* "White-adapted"
	Chromatophore	Red	Yellow	White	Black	Red	Orange	White	Black	
	Initial Pigmentary State	D	D	S–D	C	D	D	S–D	D	C
Effect of Injection of Extract	Sinus Gland	C	C	D?	D?	C	C	D	C?	D
	Eyestalk excluding Sinus Gland	C	C	C	D?	C	C	D		
	Optic Ganglia	C	C	D?					—	D?
	Medulla terminalis	C	C	C	D?	C	C	D	C?	D
	Brain	C	C	C	D?	C	C	D	C?	D
	Commissural Ganglion	C	C	D?					—	D?
	Thoracic Ganglion	C	C	D?	D?	C	C	D	C?	D

Notations: C, pigment concentration; D, pigment dispersion; S–D, semi-dispersion of pigment; ?, indefinite pigmentary response; —, no visible pigmentary response.

Extracts of Sesarma were further tested upon the chromatophores of *Paratya compressa*, a fresh-water shrimp, *Ocypoda stimpsoni*, a sea-shore crab, and *Megaligia exotica*, a sea-shore isopod, which all show remarkable color change. Concerning the physiology of color change of Paratya and Megaligia, papers by Nagano (1943) and Enami (1941) are to be referred to. For the present purpose, eyestalkless Paratya, eyestalkless Ocypoda, and "blinded" (eyes covered with a mixture of Sudan Black and balsam) and "white-adapted" (eyes covered at the dorsal aspect with the opaque mixture, and the animal kept in white container under dim light) Megaligia were employed. Equilibrium states of chromatophores in each of these test animals are summarized in the upper column of Table II. Van Harreveld's solution was employed as the physiological solution for Paratya, and Herbst's arti-

ficial sea-water for Ocypoda and Megaligia. The dose of injection was 0.01 cc. for Paratya, 0.05 cc. for Ocypoda and 0.02 cc. for Megaligia. Fifteen individuals of each species composed one lot of test animals.

In all these experiments, chromatophore responses *in vivo* or *in vitro* were observed at intervals of 15 minutes following administration of extracts of Sesarma, and were recorded by means of the conventional chromatophore indices. For the application of the indices to actual pigmentary states of chromatophores in a given area of the skin, the arbitrary method proposed by Hogben and Slome (1931) was adopted. Indices obtained with a lot of test objects at a given time were averaged to be plotted for a graph representing the time relation of chromatophore responses. This indicated the effectiveness of a given extract.

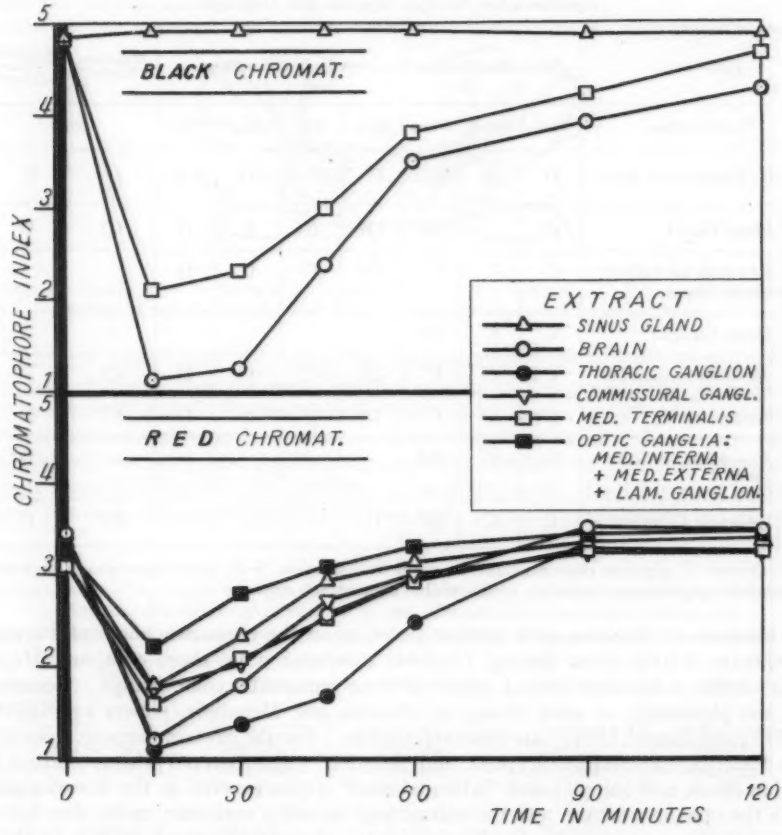


FIGURE 3. Curves showing time relations of responses of black and red chromatophores of eyestalkless *S. haematocheir*, juv. to injection of active extracts from adult *S. dehaani*. Concentration was 0.5 T.E. per 0.05 cc. in respective extracts.

Experiments and Results

Because it was concluded that in the three species of Sesarma employed the endocrine system is alike, for the sake of simplification the following account is generalized and covers all species used.

Injection Experiments upon S. haematocheir, juv.[2]

The result of injection experiments with tissue and organ extracts from adult forms of Sesarma was that only the extracts of sinus gland and nervous organs were effective in inducing definite chromatophore responses in the eyestalkless *S. haematocheir,* juv. Others, such as the extracts of stomach, intestine, hepatopancreas, heart, male genitalia, gill, muscle and hypodermis together with underlying connective tissue, were not responsible for any definite chromatophore reaction, neither was the control injection of plain van Harreveld's solution.

From the point of view of qualitative effects (cf. Fig. 3), the activities of the extracts might be arranged according to the following classification:

a) Extracts to which the red and the vermilion chromatophores were susceptible. Response: correlated pigment concentration in both chromatophores. Extracts: sinus gland, Lamina ganglionaris, Medulla externa, Medulla interna, Medulla terminalis, brain, commissural ganglion, thoracic ganglion.

b) Extracts to which the black and the white chromatophores were susceptible. Responses: pigment concentration in black chromatophores, being correlated with pigment dispersion in the white ones. Extracts: brain, Medulla terminalis.

In repeated trials of dilution experiments, the ineffectiveness of the sinus gland extract for the black and the white chromatophores was proved over a concentration range reaching as high as 5 T.E. per 0.05 cc., while a more or less significant effect was detected for the red and the vermilion cells even in a concentration as low as 0.001 T.E. per 0.05 cc. Extracts of all the principal nervous organs including brain and Medulla terminalis were effective for the red and vermilion chromatophores in various degrees, those of larger ganglia, such as thoracic ganglion, brain and Medulla terminalis, being even more effective than the sinus gland extract in the same T.E. The black and white chromatophores were affected only by extracts of brain and Medulla terminalis, the former extract exceeding the latter in effectiveness, acting even in a concentration of 0.0015 T.E. per 0.05 cc. Figure 4 shows the relation between the concentration of the brain extract and the magnitude of response of the black chromatophores, measured from time relation curves obtained by a series of dilution experiments. As is shown, the magnitude of effectiveness was exponentially proportional to the concentration of the extract, the relation between them being significantly sensitive only over a restricted range of lower concentrations.

Experiments upon Isolated Carapace Fragments of S. haematocheir, juv.

From the experiments with isolated carapace fragments obtained from eyestalkless *S. haematocheir,* juv., the qualitative activities of the active extracts of adult Sesarma were again ascertained. Observations with the carapace fragments turned

[2] Abbreviation of juvenile form.

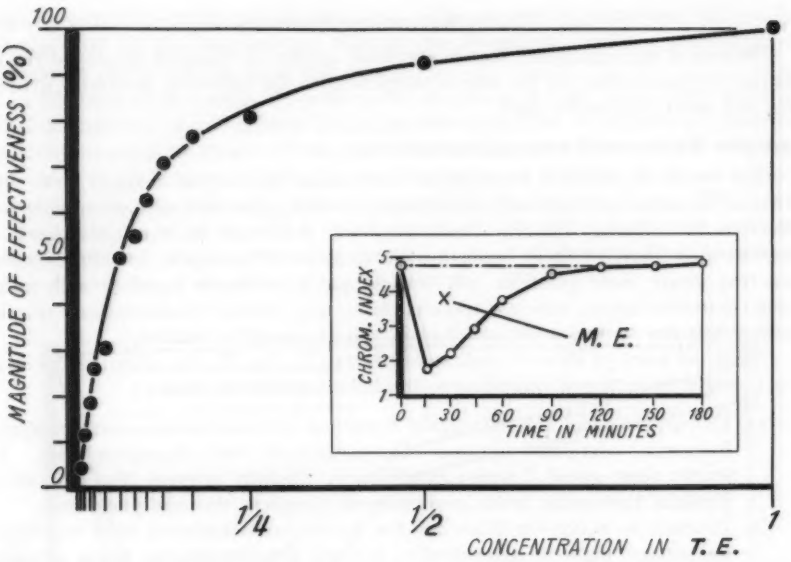


FIGURE 4. Curve showing relationship between percentage of magnitude of effectiveness and concentration of brain extract from adult *S. dehaani*. Figure inserted indicates method of estimation of magnitude of effectiveness: Area enclosed by time relation curve of response of black chromatophores and a horizontal line representing initial pigmentary state of the chromatophores was measured and designated to be magnitude of effectiveness (M.E.) of given extract. M.E. of 1 T.E. per 0.05 cc. was taken as standard value, against which percentile ratio of M.E. of respective concentration was calculated.

inside out facilitated detailed examination of the behavior of the white chromatophores, which are located in the deepest layer of the skin and are masked in various degrees when viewed from the outer surface of the carapace. The correlated responses of the cells with the black ones under the influence of the extracts of brain and Medulla terminalis were clearly demonstrated in the course of the experiments (Fig. 5).

Hormone Production by Nervous Organs

The foregoing results indicated that a kind of chromatophorotropic hormone, or a group of hormones, which, with respect to its qualitative effect, appeared to be identical with the one included in the sinus gland (sinus gland hormone), was almost universally distributed in the central nervous system of adult Sesarma. The question arose whether such a kind of hormone was originating essentially in the nervous tissue or whether it was derived from the sinus gland and stored in the former.

The question seemed to be answered in part by the fact that the effectiveness of extracts of some ganglionic tissues exceeded that of the sinus gland extract, which fact might suggest that the hormone under consideration was more concentrated in certain ganglia than in the sinus gland itself. However, definite proof of the

origin of the hormone in the nervous tissues themselves was obtained from an experiment, designed for the assay of the activity of the brain extract prepared from a lot of adult Sesarma that had been kept in the eyestalkless state for 30 days. Since the donor of the extract had been without the sinus gland, the brain extract might be reasonably concluded to be free from possible contamination of the sinus-gland hormone.

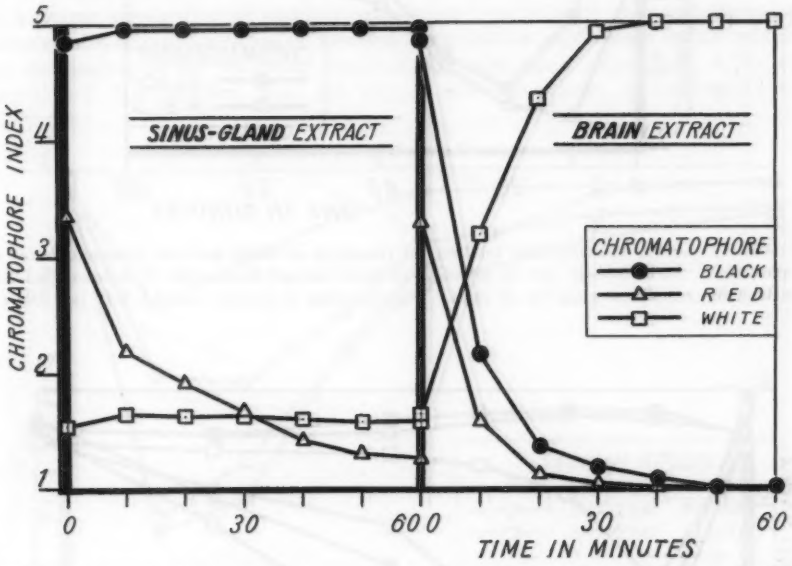


FIGURE 5. Curves showing time relations of responses of black, red and white chromatophores from eyestalkless *S. haematocheir,* juv., kept *in vitro,* to application of active extracts from adult *S. intermedia.* Concentration was 3 T.E. per 3 cc. in respective extracts.

As is shown in Figure 6, the effectiveness of the extract for the red and the vermilion chromatophores was almost comparable to that of the extract prepared from the normal animals. The same was the case with the effectiveness for the black and the white chromatophores, an observation which indicated the possible independence of the mechanism for the production of the respective hormones within the brain.

Hormone Production in Juvenile Forms of Sesarma

Injection experiments as well as experiments upon isolated carapace fragments were carried out with extracts prepared from juvenile forms of the three species of Sesarma, using the chromatophores of *S. haematocheir,* juv. as test objects. The result was that the chromatophorotropic principle known to be present in the nervous system of adult crabs was also found in juvenile forms. As to the distribution of the hormone affecting the black and the white chromatophores, it ap-

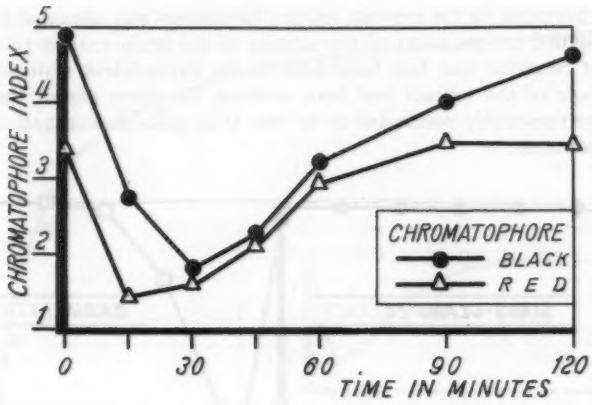


FIGURE 6. Curves showing time relations of responses of black and red chromatophores of eyestalkless *S. haematocheir*, juv. to injection of brain extract from adult *S. dehaani* that had been kept in eyestalkless state for 30 days. Concentration of extract was 0.5 T.E. per 0.05 cc.

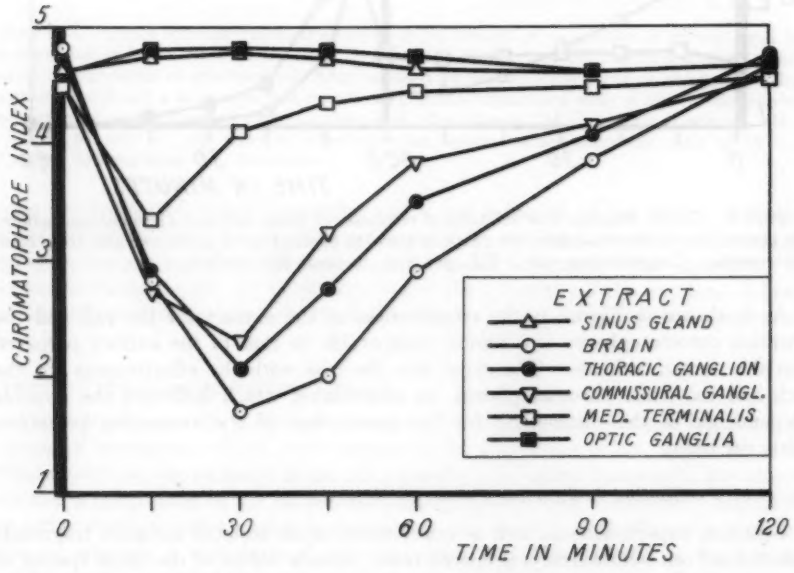


FIGURE 7. Curves showing time relations of responses of black chromatophores of eyestalkless *S. haematocheir*, juv. to injection of active extracts from juvenile *S. dehaani*. Concentration was 1 T.E. per 0.05 cc. in respective extracts.

pears to be distributed over a larger area of the nervous system in juvenile specimens than in adults, *i.e.*, in young forms not only the brain and the Medulla terminalis, but also the thoracic and commissural ganglia rendered active extracts (Fig. 7). The order of effectiveness of these extracts for the black chromatophores in the same T.E. was: brain > thoracic ganglion > commissural ganglion ≫ Medulla terminalis.

The observation that a certain incretory mechanism for the production of a kind of hormone, responsible for pigment concentration in the black chromatophores, was detected also in juvenile forms of Sesarma, suggested an explanation for the underlying mechanism of the blanching response occurring upon amputation of the eyestalks in *S. haematocheir*, juv. (cf. Fig. 1, and the account in MATERIALS AND METHODS).

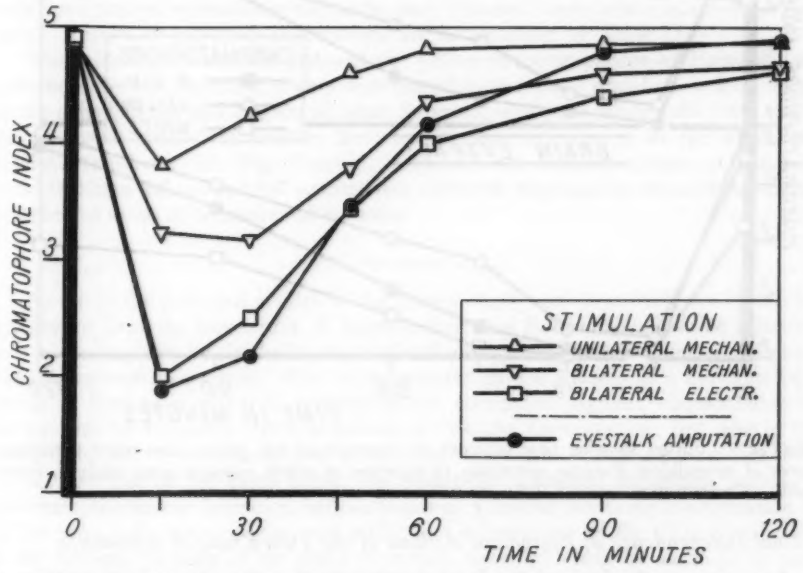


FIGURE 8. Curves showing time relations of responses of black chromatophores of eyestalkless *S. haematocheir*, juv. subjected to stimulation of stub of eyestalk.

A similar temporary blanching response was brought about by means of bi- or uni-lateral stimulation of the eyestalk-stubs of eyestalkless *S. haematocheir*, juv. (Fig. 8). Stimulation was performed either mechanically (pressing with small spherical head of a slender glass rod) or electrically (applying faradic current induced by the Harvard Inductorium connected to a 2 volts source; employing the bi- or uni-polar method). A comparison of time relation curves of reactions of the black chromatophores to respective treatments with that obtained as the result of amputation of eyestalks, indicated that all the reactions occurred under similar circumstances in the immediate surrounding of the chromatophores. In other words, it may be concluded that stimulation of the optic tract activates an incretory mechanism of the

brain or other ganglia to deliver the hormone responsible for pigment concentration in the black chromatophores.

Regarding such possible nervous control of hormone secretion by the nervous tissues, an attempt was made to inquire into the effect of acetylcholine, pilocarpine and atropine. These were injected in doses ranging from 2.5 γ to 50 γ, but none of them was responsible for any positive effect (*i.e.*, pigment concentration in the black chromatophores).

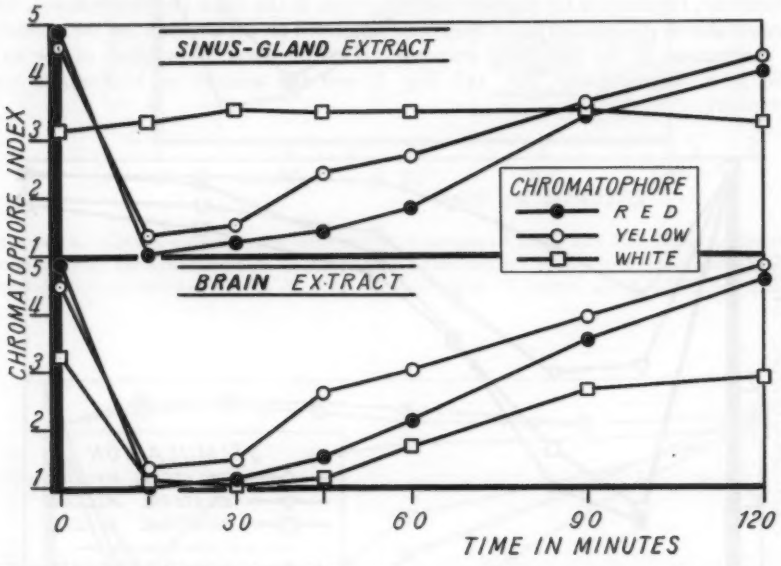


FIGURE 9. Curves showing time relations of responses of red, yellow, and white chromatophores of eyestalkless *Paratya compressa* to injection of active extracts from adult *S. intermedia.* Concentration was 0.5 T.E. per 0.01 cc. in respective extracts.

Mutual Independence of Peripheral Actions of the Two Kinds of Hormones

Assuming on the basis of the foregoing results that the sinus gland contains a hormone affecting the red and the vermilion chromatophores of *S. haematocheir,* juv., while the brain, besides containing a principle like that of the sinus-gland, also furnishes a hormone acting on the black and white cells, double injections of these extracts were tried for the purpose of learning something about any possible interference between the peripheral actions of the two hormones.

At first one of the extracts was injected, and at the end of 5 minutes the second injection of the other extract followed. In another series of experiments mixtures in various proportions of the two extracts were applied to isolated carapace fragments maintained *in vitro.*

In no case was there any significant antagonism between the two extracts. The response of the black chromatophores was always dependent upon the concentration of the brain extract, being by no means significantly affected by the co-existing

sinus-gland extract. The magnitude of response of the red and the vermilion cells was variously amplified in every instance over the range determined by the sinus-gland extract alone. This may be explained by possible summation of the effects of the hormone responsible for the red and vermilion cells present in both of the extracts applied, on the assumption that the hormone originating in the brain is identical with the sinus-gland hormone at least as to the peripheral activity.

Chromatophorotropic Activities of Extracts from Sesarma as Tested upon Paratya, Ocypoda and Megaligia

In order to obtain further information concerning the possible differentiation into two kinds of chromatophorotropic hormones in Sesarma, the activities of the effective extracts from adult forms were assayed upon the chromatophores of eyestalkless Paratya, eyestalkless Ocypoda, and "blinded" and "white-adapted" Megaligia. Results of injection experiments are summarized in Table II.

The table shows that the red and the yellow chromatophores of Paratya were influenced by the hormone widely distributed in the sinus gland as well as in all of the principal nervous organs of adult Sesarma, while the white cells were under the exclusive control of another kind of hormone originating in the brain and Medulla terminalis (cf. Fig. 9 also). However, the chromatophores of Ocypoda and Megaligia did not exhibit qualitatively different responses to the administration of different kinds of hormones of Sesarma.

Discussion

So far as the principal results of the present study are concerned, the endocrine systems of *Sesarma intermedia, S. haematocheir* and *S. dehaani* are to be classified into two categories on the basis of the distribution of the possible sources of chromatophorotropic hormones. One is represented by the sources of a hormone or a group of hormones which is responsible for correlated pigment concentration in the red and the vermilion chromatophores of *Sesarma haematocheir,* juv., and in the red and the yellow chromatophores of *Paratya compressa.* Such a kind of hormone is characteristic of the sinus gland, the universally admitted significant source of chromatophorotropic hormone, but, in Sesarma, a similar hormone is originating in all the principal nervous organs too, which fact is borne out by the wide distribution in the nervous systems of the crabs of certain incretory elements assumed to produce the active principle comparable to that of the sinus gland. Assuming tentatively the identity of the hormone originating in the nervous tissues with that in the sinus gland, I should like to propose the name S hormone (hormone characteristic of sinus gland) which comprises both the sinus-gland hormone and the one from the nervous tissues in the present cases.

The other endocrine system is represented by sources of a hormone responsible for correlated pigmentary reactions in the black (pigment concentration) and the white (pigment dispersion) chromatophores of *S. haematocheir,* juv. and pigment concentration in the white chromatophores of Paratya. In contrast to the wide distribution of the possible sources of S hormone, the range of distribution of the possible sources of this second kind of hormone is limited to particular portions of the nervous system, *i.e.,* brain and Medulla terminalis in adult forms, and brain, Medulla terminalis, thoracic and commissural ganglia in juvenile forms. In view

of the restriction of its possible source in the nervous tissues, I wish to designate such a hormone under the name N hormone (hormone essentially originating in the nervous tissues).

That a type of hormone, whose effect is qualitatively identical with the sinus-gland hormone, arises from the nervous tissues was already implied by Brown (1933), Hosoi (1934), Enami (1943), etc., who demonstrated certain measures of chromatophorotropic activity in extracts of various parts of the central nervous systems of different crustaceans. With *Uca dubia*, Enami proved that production of such a hormone in the brain and the thoracic ganglion is independent of the activity of the sinus gland, and, as already demonstrated, such is also the case with the present Sesarma.

Brown and Ederstrom (1940) offered the first concrete evidence with Crago of the occurrence in certain portions of the nervous system of a kind of hormone which is different from the sinus-gland hormone in its qualitative effect. A hormone which has thereafter been dealt with as CDH (Crago-darkening hormone) was at first detected in maximum concentration in extracts of the commissural ganglion, but later works by Brown and Wulff (1941) and Brown (1946) determined the site of essential source of this hormone to be the tritocerebral commissure. This CDH is said to be responsible for pigment dispersion in the black chromatophores distributed at the telson and uropods of Crago. Following the discovery of CDH, Brown and his associates (Brown and Wulff, 1941; Brown and Klotz, 1947) were successful in detecting and separating a second chromatophorotropic hormone (CBLH, Crago body-lightening hormone) originating likewise in the tritocerebral commissure, which is responsible for pigment concentration in the black chromatophores of the body except the telson and uropods. Antagonism was proved between these two kinds of hormones; in the absence of CBLH, CDH is effective in inducing pigment dispersion of the black cells over the whole body surface. Using Crago as test animal, Brown and Saigh (1946) examined the comparative distribution in the nervous systems of CDH and CBLH in 13 species representing Isopoda, Natantia, Astacura, Anomura and Brachyura, obtaining positive results with most of the animals. According to these authors, CBLH has in most cases wider distribution in the nervous systems as compared with CDH, which is rather restricted to certain particular portions of the nervous organs.

From the point of view of qualitative effect, what is designated as CBLH appears to be identical with the sinus-gland hormone, whose activity upon Crago black chromatophores has been determined since the pioneering work by Koller (1928). The identity of these hormones seems to be further substantiated by the work of Brown and Ederstrom (1940) who remarked that CDH and the sinus-gland hormone were mutually antagonistic. Further, the work by Brown and Klotz (1947) demonstrated that CBLH was considerably soluble in organic solvents, such as ethyl, methyl and isopropyl alcohols, in contrast to CDH, which had no significant solubility in these solvents, which fact appears to point out the similarity in chemical properties of CBLH to the sinus-gland hormone which has been demonstrated to be also considerably soluble in alcoholic solvents (Carlson, 1936; Abramowitz and Abramowitz, 1938; Abramowitz, 1940; Brown and Scudamore, 1940).

Such a consideration seems to permit a tentative generalization stating that CBLH together with the sinus-gland hormone may be dealt with under the same

category of chromatophorotropic hormones, which apparently corresponds to the present S hormone of Sesarma. Such a discussion appears to be premature in the present report, but it is tenable in that certain histological evidence proving the identity of secretory behaviors of the sinus gland and incretory elements found in the nervous systems was obtained with Sesarma, which will be published in a subsequent paper.

Assuming now that the nervous systems of crustaceans are generally charged with two kinds of chromatophorotropic hormones, as already postulated and proved by Brown, the present N hormone in Sesarma might be conceded to correspond to the said CDH, notwithstanding that CDH was reported to be absent in several brachyurans, such as *Libinia* sp., *Cancer irroratus, Carcinides maenas, Ovalipes ocellatus* and *Uca pugilator* (Brown and Saigh, 1946). As stated above, no significant antagonism was detected between S and N hormones when assayed upon the chromatophores of *S. haematocheir,* juv., but an inquiry into mutual interaction of these hormones is desirable upon the black chromatophores of Crago, before implied disparity of N hormone from CDH is taken into consideration. Brown and Saigh reported a differential distribution of CDH in the nervous systems of the crustaceans examined. In Sesarma, the distribution of N hormone in the nervous organs differs between the adult and the juvenile forms, being restricted in the former.

Hitherto no definite information was published as regards the production of chromatophorotropic hormones in the nervous tissues included in the eyestalk. Present investigation disclosed the universal occurrence of S hormone in the ganglia of the eyestalk of Sesarma and the restricted distribution of N hormone in the Medulla terminalis. On the basis of such knowledge, the data presented by Brown (1940) indicating the presence of considerable activity in the rest of the eyestalk tissues outside the sinus gland are to be attributed to the possible presence of S hormone in the ganglionic tissues in the eyestalks of crustaceans examined, *viz.,* Uca, Carcinus, Pagurus, Palaemonetes, Libinia, Callinectes and Crago. Further, Brown (1940) reported that the white chromatophores of Palaemonetes were, unlike the red ones, not significantly susceptible to the sinus-gland extract, which, according to an earlier paper by the same author (1935), were reported to be affected by the extract of the whole eyestalk. This fact appears to be explained on the assumption that N hormone is included in certain parts of ganglionic tissues in the eyestalk of the shrimp.

Thus evidence has been accumulating toward confirmation of the presence of two kinds of chromatophorotropic hormones in crustaceans, and it is to be added here that in Cambarus too McVay (1942) was cited by Brown (1944) as being successful in separating two hormones originating in the central nervous system.

Summary

1. Chromatophorotropic hormones in three species of Sesarma, *viz., S. intermedia, S. haematocheir* and *S. dehaani,* are differentiated into two broad categories of S and N hormones.

2. S hormone is responsible for pigment concentration in the red and the vermilion chromatophores of juvenile forms of *S. haematocheir,* and also for pigment concentration in the red and the yellow cells of *Paratya compressa,* while N hormone is responsible for pigment concentration in the black chromatophores and pigment

dispersion in the white ones of *S. haematocheir*, juv., and also for pigment concentration in the white cells of Paratya.

3. S hormone is distributed not only in the sinus gland but in all the principal nervous organs in both the adults and the juvenile forms of Sesarma. Occurrence of S hormone in the nervous tissues is independent of the activity of the sinus gland, suggesting the presence of a certain endocrine organization in the nervous system of the crabs.

4. N hormone is restricted to the brain and the Medulla terminalis in the adults, being widely distributed in the brain, the Medulla terminalis, the thoracic and the commissural ganglia in the juvenile forms. Production of the hormone in these ganglionic tissues is also unaffected by the activity of the sinus gland. Stimulation of the optic tract is responsible for secretion of N hormone.

5. Antagonism between S and N hormones could not be detected through the assay with the chromatophores of *S. haematocheir*, juv.

6. The discussion is especially concerned with recent information on the sources and activities of chromatophorotropic hormones originating in the nervous tissues in other species of crustaceans.

LITERATURE CITED

ABRAMOWITZ, A. A., 1940. Purification of the chromatophorotropic hormone of the crustacean eyestalk. *J. Biol. Chem.*, **132**: 501–506.

ABRAMOWITZ, A. A. AND R. K. ABRAMOWITZ, 1938. On the specificity and related properties of the crustacean chromatophorotropic hormone. *Biol. Bull.*, **74**: 278–296.

BROWN, F. A., JR., 1933. The controlling mechanism of chromatophores in Palaemonetes. *Proc. Nat. Acad. Sci.*, **19**: 327–329.

BROWN, F. A., JR., 1935. Control of pigment migration within the chromatophores of *Palaemonetes vulgaris*. *J. Exp. Zool.*, **71**: 1–15.

BROWN, F. A., JR., 1940. The crustacean sinus gland and chromatophore activation. *Physiol. Zool.*, **13**: 343–355.

BROWN, F. A., JR., 1944. Hormones in the Crustacea. Their sources and activities. *Quart. Rev. Biol.*, **19**: 32–46, 118–143.

BROWN, F. A., JR., 1946. The source and activity of Crago-darkening hormone (CDH). *Physiol. Zool.*, **19**: 215–223.

BROWN, F. A., JR. AND H. E. EDERSTROM, 1940. Dual control of certain black chromatophores of Crago. *J. Exp. Zool.*, **85**: 53–69.

BROWN, F. A., JR. AND I. M. KLOTZ, 1947. Separation of two mutually antagonistic chromatophorotropins from the tritocerebral commissure of Crago. *Proc. Soc. Exp. Biol. Med.*, **64**: 310–313.

BROWN, F. A., JR. AND A. MEGLITSCH, 1940. Comparison of the chromatophorotropic activity of insect corpora cardiaca with that of crustacean sinus glands. *Biol. Bull.*, **79**: 409–418.

BROWN, F. A., JR. AND L. M. SAIGH, 1946. The comparative distribution of two chromatophorotropic hormones (CDH and CBLH) in crustacean nervous systems. *Biol. Bull.*, **91**: 170–180.

BROWN, F. A., JR. AND H. H. SCUDAMORE, 1940. Differentiation of two principles from the crustacean sinus gland. *J. Cell. Comp. Physiol.*, **15**: 103–119.

BROWN, F. A., JR. AND V. J. WULFF, 1941. Chromatophore types in Crago and their endocrine control. *J. Cell. Comp. Physiol.*, **18**: 339–353.

CARLSON, S. P., 1936. Color changes in Brachyura crustaceans, especially in *Uca pugilator*. *Kungl. Fysiogr. Sällsk. Lund Förh.*, **6**: 63–80.

DRACH, P., 1939. Mue et cycle d'intermue chez les crustacés décapodes. *Ann. Inst. Océan., Monaco*, **19**: 103–391.

ENAMI, M., 1941. Melanophore responses in an isopod crustacean, *Ligia exotica*. I. General responses. *Jap. J. Zool., Tokyo*, **9**: 497–514.

ENAMI, M., 1943. Chromatophore activator in the central nervous organs of *Uca dubia*. *Proc. Imp. Acad., Tokyo*, **19**: 693–697.

HANSTRÖM, B., 1933. Neue Untersuchungen über Sinnesorgane und Nervensystem der Crustaceen. II. *Zool. Jahrb., Abt. f. Anat. u. Ont. Tiere*, **56**: 387–520.

HANSTRÖM, B., 1947. Three principal incretory organs in the animal kingdom. The sinus gland in crustaceans, the corpus cardiacum-allatum in insects, the hypophysis in vertebrates. Three lectures at the University of London, 1946. Copenhagen. 62 pp.

HOGBEN, L. T. AND D. SLOME, 1931. The pigmentary effector system. VI. The dual character of endocrine co-ordination in amphibian colour change. *Proc. Roy. Soc. London, Ser. B*, **108**: 10–53.

HOSOI, T., 1934. Chromatophore activating substance in the shrimps. *J. Fac. Sci. Imp. Univ. Tokyo*, **3**: 265–270.

KOLLER, G., 1928. Versuche über die inkretorischen Vorgänge beim Garneelenfarbwechsel. *Zeitschr. f. Vergl. Physiol.*, **8**: 601–612.

MC VAY, J. A., 1942. Physiological experiments upon neurosecretion, with special reference to Lumbricus and Cambarus. Not accessible, cited from Brown (1944).

NAGANO, T., 1943. Physiological studies on the pigmentary system of Crustacea. I. The color change of a shrimp *Paratya compressa* (De Haan). *Sci. Rep. Tohoku Imp. Univ., Ser. IV*, **17**: 223–245.

A SEROLOGICAL ANALYSIS OF THE SYSTEMATIC RELATIONSHIP OF THE BRACHYURAN CRAB GERYON QUINQUEDENS

CHARLES A. LEONE

Department of Zoology, University of Kansas, Lawrence, Kansas

The taxonomic relationship of the brachyuran crab *Geryon quinquedens* is open to question. Rathbun (1918) places the species in a sub-family, the Carcinoplacinae, of the family Goneplacidae. Bouvier (1940), on the other hand, lists Geryon as a genus in the family Xanthidae. Dr. Waldo Schmitt, U. S. National Museum, has indicated (personal communication to Boyden in 1935) that morphological studies do not yield a precise placement of this species in the families of the Brachyura.

Boyden (1943) conducted the first serological tests with the serum of *Geryon quinquedens*. His tests, involving among others an anti-Geryon and an anti-Menippe serum, showed reciprocal reactions which indicated as high a degree of relationship between Geryon and Menippe, the latter a member of the family Xanthidae, as either one of these showed with other brachyuran Crustacea.

This paper presents additional serological data on the systematic position of Geryon as revealed through the precipitin reaction.

Materials and Methods

Through the facilties of the Serological Museum of Rutgers University, New Brunswick, New Jersey, it has been possible to assemble serum samples of species of Crustacea which are widely scattered geographically.[1] A list of the species whose sera were used in this report to make a serological analysis of the taxonomic position of Geryon is given in Table I.

Table I

Table showing the species whose sera were compared by serological means in this paper

Species	Source
Geryon quinquedens Smith	Tortugas, Florida, U. S. A.
Menippe mercenaria (Say)	Unknown
Panopeus herbstii Milne Edwards	Unknown
Eriphia spinifrons (Herbst)	Naples, Italy
Callinectes sapidus Rathbun	Delaware Bay, New Jersey, U. S. A.
Portunus puber Linnaeus	Dinard, France
Cancer pagurus Linnaeus	Dinard, France
Pachygrapsus marmoratus Fabricius	Naples, Italy
Ocypode albicans Bosc	Tortugas, Florida, U. S. A.
Maia squinado Rondalet (Herbst)	Plymouth, England

[1] The author in his capacity as an officer of the Serological Museum in the summer of 1948 collected samples of sera from European Crustacea at marine biological stations in France and Italy. Other sera indicated in Table I were either collected personally by the author or provided from the stocks of the Serological Museum.

The methods for preparing rabbit antisera against the Crustacea hemocyanins and the technique for performing turbidimetric titrations of these antisera with antigens have been described in detail elsewhere (Boyden, 1942; Boyden and De Falco, 1943; Leone, 1949). In brief, the antisera were prepared in rabbits which received intravenously one or two series of four doubling injections of antigen. Serological comparisons were made by measuring with a Libby photronreflectometer (1938) the precipitates which developed when antigens and antibodies were mixed in varying proportions. To each dilution of a doubling dilution series of antigen was added a constant amount of antiserum. The entire reaction range from antigen excess to antibody excess was covered. The antigen-antibody mixtures were incubated for twenty minutes at 38° C. The precipitates which formed in the mixtures were then read in the turbidimeter. The areas of the resulting curves were used as a statistical index of the amount of reactivity between the particular antigen and antiserum being tested.

Experimental Results

The serological data are summarized in Table II.

Table II

Table showing serological relationships among brachyuran Crustacea. Values are given in percentages. Comparative values are read in vertical columns. Family names are given under each scientific name. The homologous test is identified as the 100 per cent reaction. Heterologous antigens react with the antisera in proportion to their degree of serological correspondence to the homologous antigen. Relationship values within 5 per cent of one another are considered to be of the same order of magnitude.

Antigens	Antisera								
	Geryon quinquedens	*Menippe mercenaria* (Xanthidae)	*Eriphia spinifrons* (Xanthidae)	*Callinectes sapidus* (Portunidae)	*Portunus puber* (Portunidae)	*Cancer pagurus* (Cancridae)	*Pachygrapsus marmoratus* (Grapsidae)	*Ocypode albicans* (Ocypodidae)	*Maia squinado* (Majidae)
Geryon quinquedens	100	35	28	59	37	45	64	14	14
Menippe mercenaria (Xanthidae)	39	100	28	54	35		64	34	13
Eriphia spinifrons (Xanthidae)	37	35	100	51	37	46	65	32	13
Panopeus herbstii (Xanthidae)	40	36	26	51	37		65	39	14
Callinectes sapidus (Portunidae)	36	36	23	100	37	48	54	30	21
Portunus puber (Portunidae)	34	35	21	62	100	51	55	30	20
Cancer pagurus (Cancridae)	26	27	18	40	25	100	68	33	16
Pachygrapsus marmoratus (Grapsidae)	16	15	15	25	20	40	100	35	16
Ocypode albicans (Ocypodidae)	15	12	10	21	25	35	64	100	13
Maia squinado (Majidae)	5	12	10	21	25	22	25	5	100

The data obtained with the anti-*Geryon quinquedens* rabbit serum reveal that:

(a) Of all the heterologous antigens, those of the Xanthidae and Portunidae react most. There is no significant difference in the reactivity of the species of these two families with the anti-Geryon serum.
(b) The serum of the cancrid crab reacted only slightly less than the sera of Portunidae and Xanthidae.
(c) Sera of the Grapsidae and Ocypodidae react with the anti-Geryon serum equally but still less than the above families.
(d) Of all the families tested, the representative of the Majidae gave the least amount of reactivity with the anti-Geryon serum.

The results obtained using the anti-*Menippe mercenaria* rabbit serum and the anti-*Eriphia spinifrons* rabbit serum were virtually the same for the heterologous antigens as the results obtained with the anti-Geryon rabbit serum. The serum of Geryon, moreover, reacted as strongly with these two antisera as did any other heterologous antigen tested. In view of this fact, and inasmuch as Menippe, Eriphia and Panopeus are all members of the family Xanthidae, the serological affinities of Geryon could place it in, or close to, this family.

The extent of the reaction of the species of the family Xanthidae, including Geryon, with antisera made against the sera of species of the Portunidae would indicate a close relationship between the two families. In fact, the heterologous Portunidae antigen did not react to any greater extent with an anti-Portunidae serum than did the sera of the Xanthidae. The antigen next most reactive with the anti-*Callinectes sapidus* rabbit serum was from the family Cancridae. These were followed by sera of the Ocypodidae, Grapsidae and Majidae, all equally reactive with the anti-Portunidae serum. The anti-*Portunus puber* rabbit serum was less discriminating than the anti-*Callinectes sapidus* rabbit serum. The former antiserum arranged the antigens which were tested into two principal groups, the one containing the Portunidae and Xanthidae, the other containing the Cancridae, Grapsidae, Ocypodidae and the Majidae. The latter anti-portunid serum set apart the other portunid crab from the Xanthidae, and also separated the Cancridae from the other families tested. It is interesting to note in connection with this antiserum that the proteins of Geryon reacted as much as the proteins of the heterologous portunid crab. This is unusual in view of the discriminating capacity of the antiserum. On the basis of this serological comparison, Geryon could be placed in the Portunidae rather than in the Xanthidae.

The anti-*Cancer pagurus* rabbit serum reacted most strongly with antigens from species in the families Portunidae and Xanthidae. Geryon's serum reacted as much with this antiserum as did the xanthid serum. Sera from species representing the families Grapsidae and Ocypodidae reacted somewhat less than those of the portunid and xanthid crabs. The antigens of *Maia squinado* reacted least of all with the anti-*Cancer pagurus* rabbit serum.

The anti-*Pachygrapsus marmoratus* serum grouped as equally reactive the sera of the species of the Cancridae, Xanthidae, and Ocypodidae. Sera of species from the Portunidae reacted slightly less, and the serum from the family Majidae the least.

Except for the sera of Maia and Geryon, which reacted less than the others, all the heterologous antigens reacted to approximately the same extent with the anti-*Ocypode albicans* rabbit serum. The writer can offer no explanation why the serum of Geryon should be distinguished in this manner from the sera of the xanthid and portunid crabs.

The anti-*Maia squinado* rabbit serum was unable to distinguish preferentially any of the heterologous antigens.

Discussion

Bouvier's classification (1940) of *Geryon quinquedens* is more generally confirmed by serological tests in this report than is the classification of Borradaile (1907) as copied and adapted by Rathbun (1918, 1937). Geryon serologically appears to be more like species in the families Xanthidae and Portunidae than like the species of any other of the families tested. Unfortunately, at the present writing, there is no true goneplacid serum available with which to compare the serum of Geryon or the sera of xanthid and portunid crabs. The relationship of Geryon to the Goneplacidae cannot be conclusively settled until these comparisons are made. The unlikelihood of obtaining the sera of the Goneplacidae, which would make the critical serological analysis possible, has prompted me to submit such data as are available. It is difficult to believe, however, that Geryon could exhibit more serological correspondence to the sera of the Goneplacidae than it has with those of the Xanthidae and Portunidae unless the sera of the Goneplacidae have an amount of serological correspondence to the Xanthidae and the Portunidae similar to that exhibited by Geryon.

The position of the family Cancridae as revealed through the serum of *Cancer pagurus* is interesting. When analyzed with the more discriminating antisera made against the sera of species from the tribe Brachyrhyncha such as the anti-Menippe and the anti-Callinectes sera, the antigens of Cancer are separated from those of species in Bouvier's tribe Corystoidea. Similarly, the anti-*Cancer pagurus* rabbit serum indicated that the xanthid and portunid antigens were more similar to those of Cancer than were the antigens of the grapsid and ocypodid crabs. In contrast to this the less discriminating anti-xanthid and anti-portunid rabbit sera, namely anti-Eriphia serum and anti-Portunus serum, classified the Cancer antigens as being of about the same order of reactivity as the sera of the grapsid and ocypodid crabs. Antisera against the proteins of *Pachygrapsus marmoratus* and *Ocypode albicans* did not give any greater reaction with the serum of Cancer than they did with the sera from species in the other families. The serological data indicates then that with appropriate antisera the proteins of *Cancer pagurus* can be shown to be more similar to the xanthid and portunid crabs than to the grapsid and ocypodid crabs.

Whether or not Bouvier was justified in placing Cancer in the tribe Corystoidea instead of the tribe Brachyrhyncha is a matter of opinion. The criteria which are used to establish different systematic categories are often debatable. In the case of serological data, homologous proteins of two species from different yet closely related tribes might exhibit greater serological correspondence than the homologous proteins of two widely separated species within a single tribe. This is not inconsistent with good systematics.

The anti-Grapsidae and anti-Ocypodidae rabbit sera did not give a greater reaction with the antigens of Geryon over the other heterologous antigens tested. The family Goneplacidae was classified by Bouvier with the Grapsidae and Ocypodidae in the group Catometopa. If this classification is correct, then Geryon is more correctly placed as a genus in the family Xanthidae or in the family Portunidae. However, as indicated above, exact serological relationship of Geryon to the Goneplacidae still remains to be determined.

Summary

1. Serological tests have revealed that the serum of the brachyuran crab *Geryon quinquedens* has a greater degree of correspondence to the sera of species in the families Xanthidae and Portunidae than to the sera of species in the families Cancridae, Ocypodidae, Grapsidae and Majidae.

2. Of the species which were tested serologically, those in the families Xanthidae and Portunidae have the closest relationship. The family Cancridae is closer to the Xanthidae and Portunidae than any other of the families tested.

3. The families Grapsidae, Ocypodidae, and Majidae are quite distinct from one another, and from the other families tested. The family Majidae is serologically least like any of the families tested.

LITERATURE CITED

Borradaile, L. A., 1907. On the classification of decapod Crustacea. *Annals and Mag. Nat. Hist.*, **19**: 459.

Boyden, A. A., 1942. Systematic serology: A critical appreciation. *Physiol. Zool.*, **15**: 109–145.

Boyden, A. A., 1943. Serology and animal systematics. *Amer. Nat.*, **77**: 234–255.

Boyden, A. A. and R. J. De Falco, 1943. Report on the use of the photronreflectometer in serological comparisons. *Physiol. Zool.*, **16**: 229–241.

Bouvier, E. L., 1940. Faune de France 37, Decapodes Marcheurs. Paul Lechevalier et Fils. Paris.

Leone, C. A., 1949. Comparative serology of some brachyuran Crustacea and studies in hemocyanin correspondence. *Biol. Bull.*, **97**: 273–286.

Libby, R. L., 1938. The photronreflectometer—an instrument for the measurement of turbid systems. *J. Immunol.*, **34**: 71–73.

Rathbun, M., 1918. The Grapsoid crabs of America. *Bull. 97, U. S. Nat. Museum.*

Rathbun, M., 1937. The Oxystomatous and allied crabs of America. *Bull. 166, U. S. Nat. Museum.*

THE ACTIVITY OF THE CONTRACTILE VACUOLE IN A SUCTORIAN (TOKOPHRYA INFUSIONUM) [1]

MARIA A. RUDZINSKA AND ROBERT CHAMBERS

Department of Biology, Washington Square College of Arts and Science, New York University, and the Marine Biological Laboratory, Woods Hole, Massachusetts

This paper deals with a study of the behavior of the contractile vacuole during several functional phases in the life of *Tokophrya infusionum*, a protozoon of the Class Suctoria.

The Tokophrya has proved to be exceptionally favorable: (a) The sedentary habit of the adult facilitates observation on the contractile vacuole during all periods of its activity. (b) The food supply can be regulated since the organism feeds only on living ciliates. (c) The contents of the prey are incorporated by being sucked out through the predatory tentacles of the host. This eliminates the complication of having water introduced, a usual accompaniment of the formation of food vacuoles in other protozoa. (d) During reproduction a parent organism persists, since Tokophrya reproduces by the formation of a succession of "embryos" in temporary brood pouches.

General Description of Tokophrya

Tokophrya, a fresh water, sessile suctorian, possesses a spheroidal or pyriform body with an average diameter of 17 to 50 micra. Two bundles of straight, motionless, sucking tentacles (5–20 in each bundle) extend, one from each side of the body (Fig. 1A). The contractile vacuole, of which there may be two, rarely three, has an average maximum diameter of 10 micra. Feeding starts as soon as the prey, generally consisting of small living ciliates, becomes attached to one or several tips of the extended tentacles. One Tokophrya may feed simultaneously on ten or more ciliates, each of which may be several times the size of the Tokophrya. In a starved Tokophrya the body is translucent and the macronucleus is then clearly visible. During feeding, the body becomes opaque and filled with numerous dark food vacuoles. With time these vacuoles become increasingly translucent and finally disappear within 24–48 hours. The capture of the swimming prey depends upon its chance contact with the tentacles of the Tokophrya. Shortly after being caught, the prey becomes motionless and, a few seconds later, granules of cytoplasm from the prey can be seen to flow through the hollow tentacles into the host. Within 5 to 10 minutes the prey progressively shrinks in size and, after 25 minutes or more, only a spherical remnant is left, which becomes detached and disintegrates. It is of interest to note that the contractile vacuole of the prey continues to pulsate until just before the prey is reduced to the discarded small remnant.

Tokophrya reproduces by endogenous budding, the bud being produced within a temporary vacuole, the so-called "brood pouch." A well-fed individual is able

[1] This work was supported through funds from the National Cancer Institute of the U. S. Public Health Service.

to produce as many as twelve embryos successively during 24 hours. The first indication of budding is the formation of the brood pouch which appears as a large triangular vacuole at the apical region of the body. After a few minutes the vacuole becomes crescentic as it progressively encircles one of the two mitotically produced

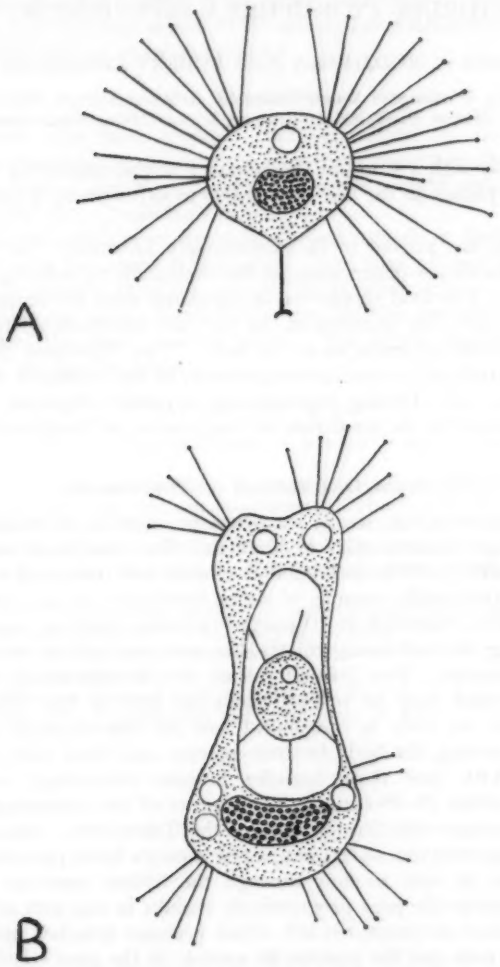


FIGURE 1. A. An adult *Tokophrya infusionum* with two bunches of tentacles, a stalk with an attachment disc, one contractile vacuole and a macronucleus. B. An abnormal case of a *Tokophrya infusionum* in which a young adult persists in the brood pouch. There are five bunches of short tentacles and five contractile vacuoles. The enclosed young Tokophrya has one contractile vacuole and five tentacles, the tips of three of which are in contact with the wall of the brood pouch.

micronuclei, together with some cytoplasm and a small portion of the macronucleus. The embryo, thus constituted, is pinched off, becomes ciliated and rotates within the brood pouch for about 10 to 20 minutes before it is ejected to the exterior. The whole process, from the time of the formation of the brood pouch until the liberation of the embryo, lasts for a little more than an hour. The liberated embryo swims for several minutes to several hours after which it becomes attached, loses its cilia and is transformed in the next few minutes into a mature form. The development and subsequent ejection of the embryo in Suctoria has been fully described by Collin (1911, 1912), Penard (1917–23), Pestel (1931), Noble (1932).

Material and Methods

The material for this study was obtained in the spring of 1948 through the courtesy of Dr. Daniel Lilly, St. John's College, Brooklyn, New York, from a pond in the vicinity of Laurelton, New York. From one of several mass cultures, 20 pure line cultures of *Tokophrya infusionum* were started. These were maintained in standing drops on microscopic slides bounded by walls of thick vaseline or a mixture of paraffin and beeswax. All the cultures were kept in large moist chambers and transfers were made weekly.

Bacteria-free cultures were obtained by transferring a free swimming embryo through several washes of autoclaved Great Bear Spring water. The ensuing progeny were maintained throughout in autoclaved water. The food supply consisted of bacteria-free cultures of *Tetrahymena geleii*. These were grown in a proteose-peptone medium. The cultures of Tokophrya were fed with Tetrahymena three times a week and were transferred to fresh media every three to four weeks.

The contractile vacuole always undergoes complete contraction. Its pulsation rate was recorded by means of a stop watch and its diameter was measured when at a maximum just prior to its periodic expulsion. These measurements were used to calculate the amount of fluid expelled over a given period of time. All studies were made at room temperature.

Experimental

The contractile vacuole during feeding

Fifty individual Tokophrya, selected from pure cultures, which had been maintained without food for 12 to 24 hours, were mounted singly in hanging drops. Into these drops were introduced one, two or three Tetrahymena as food. It was found that in every case the pulsation rate increased suddenly after attachment of the prey, remained high during feeding, and fell abruptly at the termination of the feeding. On the other hand, the maximum diameter was found to vary in different individuals. Out of the 50 examined, it was larger in two, remained the same in 24 and was smaller in 24, during than before feeding. No significant differences were obtained as to pulsation rate or diameter, irrespective of whether the individuals were fed simultaneously on two, three or four Tetrahymena.

Table I presents the values obtained in one individual which was fed with one Tetrahymena and in which the maximum diameter of the contractile vacuole was less during than before feeding. The table presents in four columns (1) the intervals, in seconds, between six successive pulsations, (2) the average maximum diameters, in micra, of the contractile vacuole, (3) values calculated from the average

TABLE I

Activity of the contractile vacuole of a Tokophrya before, during and after feeding

	Intervals in seconds between six successive systoles					Av. maximum diam. of cont. vacuole	Av. amt. fluid expelled per second	Calculated number of systoles per hour
Before feeding	175″	125″	171″	185″	180″	10.4 μ	3.52 μ^3	22
During feeding	22″	23″	24″	23″	21″	9.7 μ	21.15 μ^3	159
Immediately after feeding	45″	45″	47″	45″	46″	9.7 μ	10.48 μ^3	79

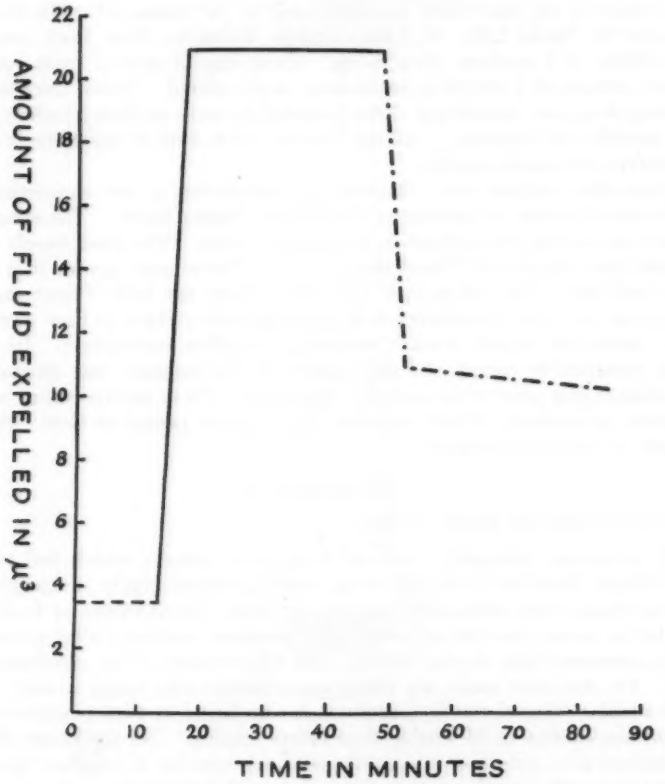


FIGURE 2. The curve represents the amount of fluid expelled by the contractile vacuole before, during and after feeding. The abscissae give the time in minutes; the ordinates, the amount of fluid in μ^3 expelled calculated for 1 sec. Data from Table I.

– – – – – – – – Before feeding
———————— During feeding
—·—·—·—·—·— After feeding

amount of fluid expelled in 1 second, and (4) the average number of systoles in one hour. From these figures it can be seen that the amount of fluid expelled by the vacuole averaged six times more during than before feeding. The amount of fluid expelled immediately after feeding was still three times as great as before feeding.

The characteristic features in these experiments are the sudden acceleration of the pulsation rate when feeding started, the increased amount of fluid expelled during and a gradual deceleration of the rate after feeding.

Figure 2 presents graphically this phenomenon. To be noted is the steepness of the upward slope representing the increase of fluid expelled when feeding has begun. The following horizontal part of the curve shows the constancy in the activity of the contractile vacuole during feeding. The subsequent rapid fall of the curve represents the decrease when feeding had ceased. Following this the curve drops gradually and, 35 minutes after feeding, is still far from the low level before feeding. Measurements were continued up to several hours after feeding at which time the level was still appreciably high.

Table II presents a striking fact that the increase in volume of the Tokophrya during feeding is appreciably less than might be expected on the basis of the volume of the food ingested. One Tetrahymena is about two to ten times larger than one

TABLE II

Volumes of three spherical Tokophrya before and after feeding. The three individuals were fed with one, two and three Tetrahymena respectively

	Fed on one Tetrahymena	Fed on two Tetrahymena	Fed on three Tetrahymena
Volume before feeding	12,000 μ^3	9,000 μ^3	11,000 μ^3
Volume after feeding	22,000 μ^3	25,000 μ^3	38,000 μ^3
Increase in volume	10,000 μ^3	16,000 μ^3	27,000 μ^3

Tokophrya. Provided the entire mass of the prey is incorporated, the Tokophrya, after having ingested one Tetrahymena, should increase in size by 200 to 1000 per cent instead of only about 80 per cent as actually occurs. This appears in the table which gives the volumes in cubic micra of each of three spherical Tokophrya before and after being fed with one, two and three Tetrahymena respectively. The table shows that in all three cases the increase in volume was definitely less than it should be when one considers the volume of the prey incorporated. The Tetrahymena is irregularly cucumber-shaped and its volume had to be estimated by considering it as an ellipsoid with long and cross axes of 60 and 24 micra respectively. The calculation gave a figure of 47,000 micra.

Calculations were also made of the volume of material actually incorporated by measuring (1) the time taken for the ingestion to take place, (2) the rate of movement of the granules through the tentacles from the prey to the host, and (3) the number, diameter and length of the predatory tentacles. The value obtained from these calculations accorded closely with that of the actual volume of the prey.

The difference between the expected and the actual increase in the volume of the host, as shown in Table II, can be accounted for by the amount of fluid expelled from the host by its contractile vacuole. This difference approximates the value

calculated from the estimated volume of the ingested Tetrahymena (44,000 μ^3) and the amount of excess fluid expelled by the contractile vacuole of the Tokophrya during feeding (roughly 40,000 μ^3) minus the amount of fluid (6,300 μ^3) expelled before feeding. The figure, 10,300 μ^3, which is 44,000 − (40,000 − 6,300), closely approximates the value of 10,000 μ^3, shown in Table II, of the Tokophrya fed with one Tetrahymena.

The contractile vacuole during reproduction

Experiments were performed to determine the effect of reproduction on the activity of the contractile vacuole. Unfed Tokophrya were used. Some were young adults kept without food since metamorphosis, others were adult Tokophrya which had been maintained without food for at least 12 hours.

It was found that the process of reproduction greatly accelerated the pulsation rate of the contractile vacuole and the amount of fluid expelled. This acceleration was progressive, starting from the time the brood pouch could be seen beginning to form, and reaching a maximum when the ciliated embryo was being completed as an isolated individual within the brood pouch. From that time on, the activity of the contractile vacuole of the mother decelerated, fell greatly by the time the embryo was expelled and still more so when the expulsion was complete. This last value was below the level determined at the beginning of the experiment and is to be interpreted as that of the individual before the reproductive process had started. At the time when the observations were made, the brood pouch had already begun to form and, therefore, the activity of the contractile vacuole presumably was already on the increase.

TABLE III

Activity of contractile vacuole in a Tokophrya during reproduction

Reproductive stages	Time in minutes	Intervals in seconds betw. six successive systoles					Av. maximum diam. of contr. vac. in μ	Av. amt. fluid expelled, μ^3/1 sec.	Calculated number of systoles per hour
Brood pouch initiated	0	86	87	90	91	82	11.7	9.66	41
Cilia of embryo in action	15	82	80	74	67	67	11.7	11.50	49
Contractile vacuoles of embryo in action	25	44	40	37	35	35	10.4	15.50	95
Embryo being completed and starting to rotate	30	27	25	25	27	29	10.4	21.82	133
Embryo being expelled	67	55	59	60	60	60	9.8	8.36	61
Expulsion completed	75	80	90	107	120	120	9.8	4.79	35

Table III presents figures for the intervals, in seconds, between the pulsations and the maximum diameter of the contractile vacuole during successive stages of the reproductive process, *viz.*, (a) the initiation of formation of the brood pouch, (b) the beginning of the ciliary activity of the forming embryo, (c) the time when the contractile vacuoles of the embryo came into action, (d) the separation of the embryo into an isolated individual, (e) the beginning and (f) the completed expulsion of the embryo.

Figure 3 represents graphically this phenomenon. The upward slope of the curve represents the increasing vacuolar activity from the moment the brood pouch

started to be formed until the peak is reached when the embryo was fully developed. The subsequent fall represents the decrease of the vacuolar activity after the completed growth of the embryo.

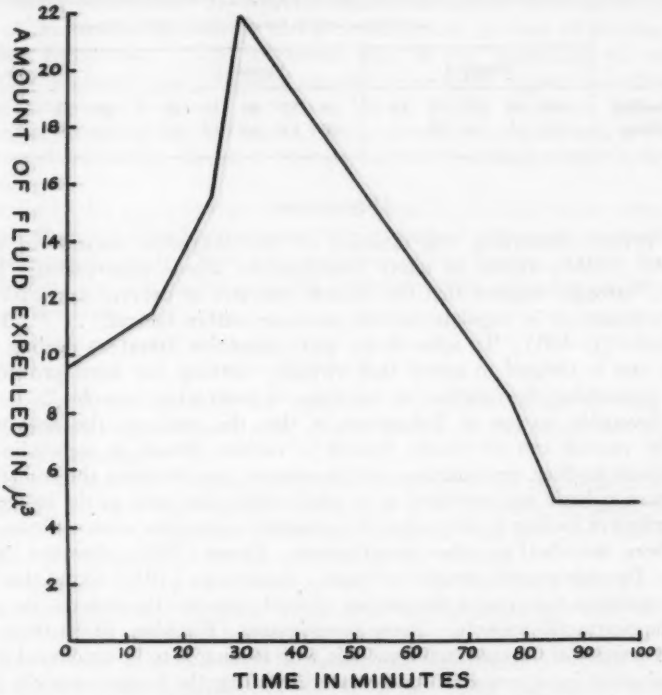


FIGURE 3. Successive changes in the amount of fluid expelled during the reproductive period. The abscissae give the time in minutes; the ordinates, the amount of fluid in μ^3 calculated for 1 sec. Data from Table III.

The contractile vacuole in individuals with abnormal reproduction in which the embryo underwent metamorphosis but persisted as an adult within the brood pouch

The contractile vacuole of these abnormal Tokophrya (Rudzinska, in press) was studied in order to ascertain whether the presence of the young adult, completely enclosed within the mother and with its tentacles attached to the wall of the brood pouch, had any effect on the activity of the contractile vacuole of the mother. In the six abnormal Tokophrya studied, it was found that the mother developed large, supernumerary contractile vacuoles which pulsated at a faster rate than normal. This is shown in Table IV, which is to be compared with Table I.

Of these abnormal individuals, four had been kept unfed. The remaining two were fed and in these the effect of feeding was evident by the still greater acceleration of the activity of the contractile vacuole.

TABLE IV

Activity of three contractile vacuoles in a Tokophrya with a young adult persisting within the brood pouch

	Intervals in seconds between six successive systoles														
	Vacuole 1					Vacuole 2					Vacuole 3				
Before feeding	50	48	45	45	48	55	52	50	53	50	60	55	55	58	55
After feeding	13	13	10	10	12	10	12	10	12	12	13	10	10	12	10

DISCUSSION

In a review concerning the function of the contractile vacuole in protozoa, Weatherby (1941) refers to many investigators whose observations, he states (p. 430), "strongly suggest that the vacuole operates to prevent excessive dilution of the cytoplasm, or to regulate osmotic pressure within the cell. . . ." However, he concludes (p. 430): "In spite of the quite extensive literature dealing with the question, one is obliged to admit that virtually nothing has been proved beyond question concerning the function or functions of contractile vacuoles."

The favorable feature of Tokophrya is that the study of the activity of the contractile vacuole can be closely related to various phases of metabolic activity. These include feeding, reproduction and an unusual case in which the embryo underwent metamorphosis and persisted as an adult within the body of the mother.

The effect of feeding in increasing the pulsatory rate of the contractile vacuole has already been described by other investigators. Pestel (1931) observed this in the suctorian, *Dendrocometes paradoxus* Stein. Andrejewa (1931) found that in *Paramecium caudatum* the greater the number of food vacuoles, the faster is the pulsation rate of the contractile vacuole. Some investigators (Kitching, 1938) claim that the increased activity of the contractile vacuole with feeding is to be accounted for by the amount of water incorporated from the exterior during the formation of the food vacuole. However, such an explanation is excluded in Tokophrya since the only material incorporated is the contents of the prey. If the fluid expelled is only that taken in unchanged from the Tetrahymena it would be expected that when feeding is completed the pulsation rate should return soon to the previous rate. However, this does not occur. The contraction rate falls only gradually and still is appreciably high for as long as several hours after feeding. This indicates the possibility of another factor responsible for the persisting high rate of output, namely, an increase in the metabolic activity of the Tokophrya. Such a relation is supported by the findings of Shumway (1917), Riddle and Torrey (1922) and Flather (1919) that thyroxin, adrenalin, pituitary and pineal gland extract accelerate the pulsation rate of the contractile vacuole. The correlation between the activity of the contractile vacuole and increased metabolism is also suggested in those cases of Tokophrya in which the young adult persisted virtually as a parasite within the brood pouch of the mother. The increased number and rapid contraction rates of the contractile vacuoles in these mothers may be accounted for by the metabolic activity of the imprisoned, parasitic offspring. The contractile vacuoles of the mother evidently are doing double duty for herself and for her enclosed adult offspring.

The increased rate of pulsation during reproduction has also been described in the literature. Unger (1925, 1926) studied the activity of the contractile vacuole of *Paramecium caudatum, P. aurelia* and *P. calkinsi* in connection with their division rate and during endomixis. He found a relation between a reduction in the pulsatile rate of the contractile vacuole and a reduction in the rate of division and of endomixis. Dimitrowa (1928) observed that at the beginning of mitosis in *Paramecium caudatum* two additional contractile vacuoles appear and claimed this to indicate increased excretory activity. Pestel (1931) observed that the first sign for endogenous budding in *Dendrocometes paradoxus*, a suctorian, is the formation of a small contractile vacuole in that part of the cytoplasm which is destined to be the embryo.

A feature of this paper is that it has been possible to show in the same species, Tokophrya, three kinds of functional activity to each of which the contractile vacuole responds specifically. These are a response (1) to feeding, (2) to the process of reproduction, and (3) to the parasitic existence of the offspring enclosed within the body of the host.

Summary

1. Evidence is presented for the existence of a close relation between the behavior of the contractile vacuole and several phases of the functional activity of *Tokophrya infusionum*.

2. When the process of feeding starts the pulsation rate of the contractile vacuole rises suddenly, remains high during feeding and falls gradually on cessation of the feeding process. It is significant that the pulsation rate remains comparatively high for several hours after the termination of the feeding.

3. During reproduction the pulsation rate of the contractile vacuole increases as soon as the endogenous budding starts, rises and remains high until the ciliated embryo has become separated within the brood pouch. This moment is the high peak of the vacuolar activity. The pulsation rate subsequently begins to decrease and falls greatly upon expulsion of the embryo. After expulsion, which lasts several minutes, the pulsation rate of the contractile vacuole drops still lower.

4. Unusually accelerated activity of the contractile vacuole was noted in those cases in which metamorphosis of the embryo occurred within the parent and the resulting young adult persisted with the tips of its tentacles attached to the wall of brood pouch.

LITERATURE CITED

Andrejewa, E. W., 1931. Zur Frage über die physikalisch-chemische Bestimmung der Korrelationen einiger physiologischen Processe bei *Paramaecium caudatum*. *Arch. f. Protistenk.*, **73**: 346–360.

Collin, B., 1911 and 1912. Étude monographique sur les Acinitiens. *Arch. de Zool. Exp. et Gen. 5e ser.*, **8**: 421–497 and **51**: 1–457.

Dimitrowa, A., 1928. Untersuchungen über die überzähligen pulsierenden Vacuolen bei *Paramaecium caudatum* Ehrbg. *Arch. f. Protistenk.*, **64**: 462–478.

Flather, M. D., 1919. The influence of glandular extracts upon the contractile vacuoles of *Paramecium caudatum*. *Biol. Bull.*, **37**: 22–39.

Kitching, J. A., 1938. Contractile vacuoles. *Biol. Revs.*, **13**: 403–444.

Noble, E. Alden, 1932. On *Tokophrya lemnarum* Stein (Suctoria) with an account of its budding and conjugation. *Univ. Calif. Publ. Zool.*, **37**: 477–506.

PENARD, E., 1917–1923. Études sur les Infusoires tentaculifères. *Mém. de la Soc. de Physique et d'Hist. Nat. de Genève,* **39**: 131–229.

PESTEL, B., 1931. Beiträge zur Morphologie und Biologie des *Dendrocometes paradoxus* Stein. *Arch. f. Protistenk.,* **75**: 403–471.

RIDDLE, M. C. AND H. B. TORREY, 1922. The physiological response of Paramecium to thyroxin. *Anat. Rec.,* **24**: 396.

RUDZINSKA, M. A. An abnormal type of development in the life cycle of a Suctorian (*Tokophrya infusionum*). (In press.)

SHUMWAY, W., 1917. The effect of a thyroid diet upon Paramaecium. *J. Exp. Zool.,* **22**: 529–563.

UNGER, W. B., 1925. The relation of contractile and food vacuoles to rhythms in Paramecium. *Proc. Soc. Exp. Biol. Med.,* **22**: 333.

UNGER, W. B., 1926. The relationship of rhythms to nutrition and excretion in Paramecium. *J. Exp. Zool.,* **43**: 373–412.

WEATHERBY, J. H., 1941. The contractile vacuole. *In* Protozoa in Biological Research, Calkins, G. N. and F. M. Summers, Columbia University Press, New York, 404–447.

THE MARINE SAND-DWELLING CILIATES OF CAPE COD

E. FAURÉ-FREMIET

Collège de France, Paris, and Marine Biological Laboratory, Woods Hole, Mass.

Marine Sands and Psammophilous Ciliates

It is well known that the marine sands between the tidemarks, or deeper along the seashore, constitute some quite peculiar and interesting biotopes. With regard to their fauna, one can distinguish the sand-boring animals of large or small size and, on the other hand, the very minute ones which live in the intergranular spaces of the sand and constitute an "interstitial" microfauna.

This interstitial microfauna, or mesopsammon, includes, besides numerous copepods, nematodes, gastrotrichs, etc., many protozoa and most particularly numerous ciliates. Kahl (1933, 1935) has recorded more than 80 ciliate species, belonging to about 20 different families, from the sandy bottoms of Kiel and Helgoland.

I found many of these infusorian species and some new ones in the sand of the tidal zone of different beaches in Brittany (Fauré-Fremiet, 1950). This result ties up with some other dispersed data and agrees with a general statement of Calkins (1933) who emphasizes the cosmopolitan distribution of the marine littoral protozoan species. But, from an ecological point of view, it can be noted that the size of the intergranular spaces acts as a selective factor in the distribution of the interstitial ciliates.[1]

The intergranular spaces may be roughly estimated from the dimensions of the solid grains which bound them; a comparison of the granulometric curves plotted for different kinds of sand with the associated interstitial fauna leads one to distinguish in the fauna two different types of ciliates.

If the mean size of the sand grains exceeds 0.4 mm., the interstitial ciliate fauna includes some characteristic sand-living species and some occasionally sand-dwelling infusorians which are much more numerous. The characteristic sand-living species are generally characterized by their small or medium size, their flattened shape, and their jerky motion, with alternately very fast swimming and sudden stopping when in contact with solid surfaces. The occasional sand-dwellers, quite varied in shape and size and usually widespread along the seashore, display a common but more or less accentuated thigmotactic behavior. All these together constitute, in the medium and coarse sands, what I have called the mesoporal infusorian fauna (Fauré-Fremiet, 1950).

If the mean size of the sand grains falls between 0.1 and 0.3 mm., their interspaces shelter some very peculiar species of ciliates, generally thread-like or ribbon-like, often as long as one or two millimeters, always strongly thigmotactic. These

[1] In a previous paper (Fauré-Fremiet, 1950) are discussed some of the physical and chemical conditions which depend on the enormous development of the solid-liquid interfaces of the sand; they probably play an important role in interstitial life of the sand-dwelling ciliates.

characteristic sand-dwelling species constitute what I have called the microporal infusorian fauna.

It must be said, however, that some ciliates of the microporal type can live as well in the coarse as in the fine sand, and these species may be called euryporal.

Finally, if the mean size of the sand grains is below 0.1 mm., or if a coarser sand is so muddy that the intergranular spaces are filled with very minute organic particles, the porosity becomes incompatible with the interstitial life of any species of ciliates.

Numerous observations made on different beaches and sandy flats of the Cape Cod region during the months of June and July, 1950, offer new evidence of the cosmopolitan distribution of many psammophilous ciliate species, and of the general significance of the two types of infusorian interstitial fauna.

Technique

The superficial layers of wet sand exposed at low tide were collected with a spoon, to a depth of 2 to 10 millimeters, and put in some convenient bottle or carton, with just a little sea water. At the laboratory, the samples were transferred to large fingerbowls, covered with sea water and allowed to stand overnight.

In order to observe the ciliates, a small quantity of sand, taken from one sample with a spatula or a pipette, was put in a Petri dish and gently agitated with sea water to which a few drops of a 12 per cent $MgCl_2$ solution were added.[2]

Then the sand was gathered at one side of the dish and the water examined under a low-power binocular microscope. The psammophilous ciliates, now swimming or gliding on the glass bottom of the dish, are easily picked up with a capillary pipette and transferred to a slide for a more careful examination, or placed in small covered dishes with normal sea water and sand grains for later examination.

The granulometric analyses of three different types of sand were made at the Woods Hole Oceanographic Institution. The corresponding curves were plotted for the very fine sand of the Barnstable Harbor flats, and for the two coarse sands from Tarpaulin Cove and Nobska beaches (Fig. 1).

A rough micrometric estimation of the mean diameter of the grains was quite sufficient for the comparative study of many other samples.

Mesoporal Infusorian Fauna

The sands of almost all the beaches around Woods Hole shelter ciliate species of the mesoporal type.

The samples examined were collected in several places on different beaches at Penzance, Woods Hole (M. B. L. beach), Sippiwisset, Quisset, Gansett and Nonamesset along the Buzzards Bay coast, and at Tarpaulin Cove, Nobska, Falmouth, and Great Pond along the Vineyard Sound.

In every case the granulometric measurements show that the size of most of the sand grains lies roughly between 0.3 mm. and 1.0 mm. These sands are quartzous, with very few mica flakes, and contain, with few or no mud particles, a variable amount of organic residues.

[2] See Fauré-Fremiet (1950). Magnesium chloride, which narcotizes many small metazoans rapidly, decreases the thigmotactic adherence of the ciliates, and hence releases them from the sand particles, without a sensible alteration of their motility.

In these somewhat coarse sands, the ciliates are generally neither abundant nor very varied; they belong most often to diatom-ingesting species.

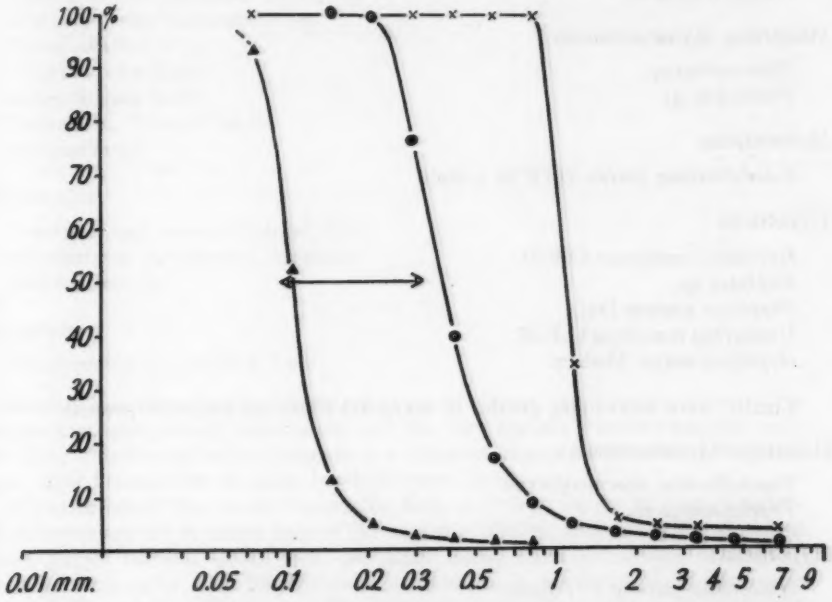


FIGURE 1. Granulometric characteristics of sands collected on three different beaches of Cape Cod. Cumulative curves; abscissa: diameter, in millimeters, of the sand grains (logarithmic scale); ordinate: weight per cent of the sand grains larger than the corresponding diameter. ▲ Barnstable Harbor flat; ● Tarpaulin Cove; × Nobska beach.

The typically mesoporal species observed are the following:

Holotricha Gymnostomata:

Mesodinium pulex, f. *pupula* Kahl
Placus (*Thoracophyra*) *buddenbrocki* Sauerbrey
Remanella margaritifera (?) Kahl

Oligotricha:

Strombidium sauerbreyae Kahl

Hypotricha:

Discocephalus rotatorius Ehrb.

The other species, widely distributed along the seashore, belong occasionally, but not specifically, to the mesoporal fauna; the following are frequently encountered:

Holotricha Gymnostomata:

Lacrymaria sp.
Coleps tesselatus

Holotricha Hymenostomata:

Pleuronema sp.
Frontonia sp.

Heterotricha:

Condylostoma patens (O.F.M.) Stein

Hypotricha:

Epiclintes ambiguus O.F.M.
Euplotes sp.
Diophrys scutum Duj.
Uronychia transfuga O.F.M.
Aspidisca major Madsen

Finally, some sand-living ciliates of euryporal character are often present:

Holotricha Gymnostomata:

Trachelocerca phaenicopterus
Trachelocerca sp.

Hypotricha:

Holosticha fasciola (?) Kahl

It is apparent that these different vegetarian species seem to be living quite independently of one another. Their quantitative distribution appears quite variable and unequal, not only from one beach to another but also along the same beach, between two areas only a few meters distant and showing the same physical appearance. Sometimes a local population of one of these species may be fairly abundant, probably in connection with the local growth of some species of diatom or bacteria utilized as food. This local growth is conditioned itself, perhaps, by some slight local difference in the intensity of light or the tidal agitation.

Microporal Infusorian Fauna

Some of the special conditions needed by the ciliates of the microporal type are found in the sand of the Barnstable Harbor flats, on the south coast of Cape Cod Bay. There the sand is very fine, slightly muddy, closely packed, and inhabited by many burrowing invertebrates, such as clams and worms.

The ciliates seem to be confined to the superficial layer of clear gray sand, sometimes reddish spotted by purple bacteria, and covering a deeper black sand rich in organic decomposition products.

The size of the sand grains varies from 0.05 mm. to 0.2 mm. In many samples collected on the flats exposed at low tide, there was found a more or less abundant and varied association of the following characteristic microporal species:

Holotricha Gymnostomata:

Helicoprorodon (*Chaenea*) *gigas* Kahl
Centrophorella fasciolata Sauerbrey
Centrophorella lanceolata n. sp.
Geleia simplex n. sp.
Geleia decolor Kahl
Geleia fossata Kahl
Geleia orbis Fauré-Fremiet
Remanella sp.

Heterotricha:

Condylostoma remanei Spiegel, Kahl
Blepharisma clarissimum Anigstein
Blepharisma sp.?

Hypotricha:

Urostrongylum caudatum Kahl

Besides this very specific microporal fauna of ciliates, one observes some of the euryporal species already mentioned, and the very curious *Parablepharisma pellitum* Kahl; this is much more abundant in a different biotope, such as the felting of a dense algal littoral flora in some brackish-water ponds.

It can be noted that on the Barnstable flats, as well as on the Brittany beaches, the different species of Geleia behave like saprobic ciliates; they are fairly abundant where purple bacteria grow. On the other hand, different species of Centrophorella, Remanella and Blepharisma are commonly encountered in the same biotope with Geleia, and they may also be found in clean fine sand and occasionally in the somewhat coarse sand of the open beaches, with some of the characteristic species of the mesoporal infusorian fauna.

Comparative Study of the Ciliate Interstitial Fauna

According to the results above, the mesoporal and the microporal infusorian fauna show respectively the same characteristics in the littoral sands of Cape Cod and in those of Brittany. However, the mean size of the sand grains available for each of the two different interstitial faunas seems to be slightly lower on Cape Cod than on the Brittany coasts; this may be due to the presence in Brittany beaches of numerous mica flakes which assure a more compact packing of the sand grains than the almost pure quartzous particles of the Cape Cod sands. In other words, with equal diameter of sand grains, the interspaces are larger between angular particles than between flat and angular particles mixed together; it is known that the dimensions of these spaces act as an important ecological factor in the distribution of ciliates (Fauré-Fremiet, 1950).

As a matter of fact, among the different Cape Cod sandy shores examined, the typical microporal infusorian fauna was only encountered in sands of the Barnstable Harbor flats. Very fine, slightly muddy, filled with numerous sand-boring animals such as annelids and clams, rich in organic residues and in sulfur bacteria, this sand resembles closely in its microfauna the sand of one beach studied in

Brittany, namely, the beach of Mousterlin, near Concarneau (Fauré-Fremiet, 1950). However, Barnstable Harbor shows some estuarine characteristics, such as some tidal variations of the sea water salinity.

Considering the cosmopolitan character of the marine littoral ciliates, the data gained from study of the Cape Cod sandy shores need some discussion. Many of the ciliates found there can be identified without hesitation, and belong to species already described, and found in the European beaches or sandy grounds. Some others appear very closely related to known species. Finally, some seem to be new species.

The following species in the sands of the Cape Cod region were positively identified with those described in the Brittany coast (Fauré-Fremiet, 1950) and the Bay of Kiel (Kahl, 1933, 1935):

Holotricha Gymnostomata:

Mesodinium pulex f. *pupula* Kahl
Placus (*Thauracophrya*) *buddenbrocki* Sauerbrey
Helicoprorodon (*Chaenea*) *gigas* Kahl (Fauré-Fremiet, 1950)
Centrophorella fasciolata Sauerbrey, Kahl
Coleps tesselatus Kahl

Heterotricha:

Condylostoma patens O.F.M.
Condylostoma remanei Spiegel
Blepharisma clarissimum Anigstein
Parablepharisma pellitum Kahl

Hypotricha:

Epiclintes ambiguus O.F.M.
Urostrongylum caudatum Kahl
Discocephalus rotatorius Ehrb.
Diophrys scutum Duj.
Uronychia transfuga O.F.M.

Among these ciliates, *Discocephalus rotatorius* requires special mention; this apparently uncommon infusorian, first described by Ehrenberg from the seashore of the Sinai peninsula, was again observed by Sauerbrey (1928) on the sand grounds of the Bay of Kiel. The form found in the coarse sand of Tarpaulin Cove shows not only the same morphological characteristics, but also the same physiological peculiarity reported by Sauerbrey, namely, the ingestion of small sand particles.

Some species, such as *Condylostoma patens*, show great variability of shape and size, but not of structure; however, this does not make identification difficult. On the other hand, it is hard to decide for some other species whether the differences observed between populations living in different regions are of specific value, or merely define some geographic varieties, or result from poor descriptions in the the literature.

Among the Holotricha, *Geleia orbis*, very abundant in the sand of Barnstable Harbor, seems to be more flattened and more thigmotactic than the Brittany form

(Fauré-Fremiet, 1950), but one does not observe any significant differences between these, either as to size (up to 1.5 mm. in full extension) or structure.

Geleia decolor Kahl is apparently identical with the Brittany species which I described under this name, but both are slightly smaller than the Kahl species and differ perhaps from his by the presence of a little anterior and preoral pit.

Among the Heterotricha a species of Blepharisma with a beaded macronucleus, very common in Barnstable Harbor, should be considered as *B. clarissimum* forma *arenicola* Kahl; it is smaller than the latter form, however, being 275 to 300 micra as opposed to 450 micra (according to Kahl). It seems to be similar to a form observed by Dragesco (personal communication) in the sand at Roscoff. On the other hand, *B. clarissimum* Anigstein is so well characterized by its cytoplasm (refringent, transparent and hyaline, like that of an Opalina) that the so-called "forma arenicola" must be considered as a distinct species.

Among the Hypotricha, a microporal Holosticha with numerous macronuclear beads is shaped nearly like *H. extensa* Kahl, with two frontal cirri; it is a little larger than Kahl's species (about 300 micra *contra* 140 to 240 micra) and possesses four or five transverse cirri like *H. fasciola* Kahl, as against 6 to 7 as in *H. extensa.* But the latter species is narrower and possesses three frontal cirri at least.

A mesoporal Aspidisca, measuring about 50 to 55 micra, resembles very closely *A. major* Madsen, in its two elongated macronuclear pieces and its five transverse cirri (as opposed to 6 for *A. binucleata* Kahl, and 11 to 12 for *A. polystyla* Stein). However, the Madsen species is larger: 60 to 90 micra.

Three presumably new species need now a more detailed description.

New Species

Centrophorella lanceolata n. sp. (Fig. 2)

The enigmatic genus Centrophorella (see Kahl, 1933, 1935 and Fauré-Fremiet, 1950), first described by Sauerbrey under the name Kentrophoros, probably belongs to the family Amphileptidae, according to Kahl.

The different species are strongly thigmotactic, very fragile and difficult to examine *in vitro.* The use of $MgCl_2$ solution shows that they are much more frequently represented in the microporal interstitial fauna than it was supposed at first. I did not observe in the fine sand of Barnstable Harbor the very large species *C. fistulosa* Fauré-Fremiet of the Brittany coast, but I found, besides *C. fasciolata* Sauerbrey, a new one which I call *C. lanceolata* n. sp. It seems highly probable that it can be found elsewhere, and a form recently observed at Roscoff, in Brittany, by Dragesco (personal communication) is perhaps the same.

Centrophorella lanceolata is a very flattened and ribbon-like ciliate, measuring about 460 to 520 micra in length, 40 micra wide and 5 to 6 micra thick. It tapers at the ends; the anterior part is slender, with a sort of neck preceding the buccal edge, curved on the right side and bearing a row of long cilia. The posterior end is pointed.

The ventral surface is ciliated, and the dorsal one is covered with an accumulation of dark sulfur bacteria, except at a narrow lateral margin at the neck and at the tail.

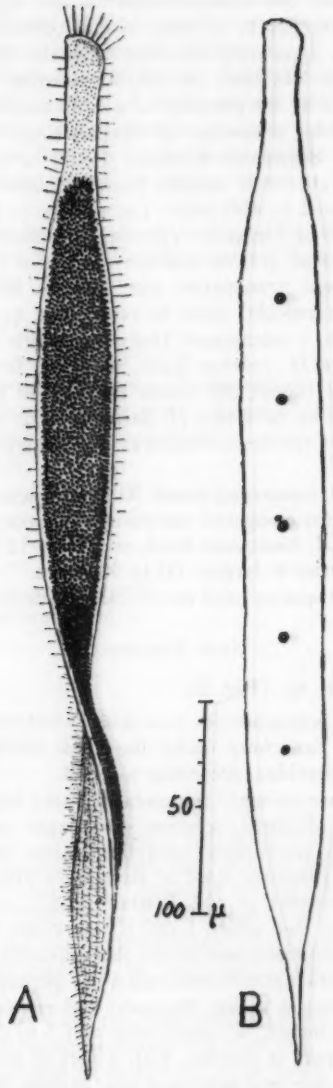


FIGURE 2. *Centrophorella lanceolata* n. sp. A: View of the living infusorian, showing the dorsal brush of sulfur bacteria. B: Outline of the ciliate showing, after staining, the size and the position of five macronuclei and one (probably) micronucleus.

The associated sulfur bacteria, rod-shaped and all of the same length, measure about 10 micra; they are attached to the dorsal surface of the ciliate infusorian body by one end, and arranged in a parallel manner like the bristles of a brush. That is a characteristic of the so-called Caulobacteria—the genus Pasteuria (Metschnikoff), for example. As is the case among others of this group, they divide longitudinally.

The dark appearance of the bacteria of *C. lanceolata* is due to the presence in their cytoplasm of highly refractile sulfur granules; in fact, these bacteria are really colorless, as are those of *C. fistulosa,* whereas those of *C. fasciolata* show a pink color.

It is known that the nuclei of the Centrophorella (Kahl, 1933, 1935; Fauré-Fremiet, 1950) are very small; *C. lanceolata* shows five to six globular macronuclei about 4 micra in diameter, and some smaller micronuclei.

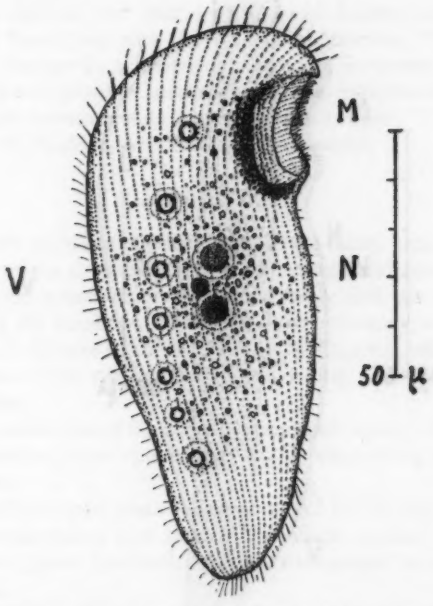


FIGURE 3. *Remanella obtusa* n. sp. View of the ciliated right side. M, mouth; N, double macronucleus with one micronucleus; V, Müller's vesicles with refringent bodies.

The association of the Centrophorella with the Caulobacteria is a perfectly constant character; in each case these bacteria are sulfur bacteria, but they differ in size and color with each species of Centrophorella. It would be interesting to define the nature, specific or adaptively induced, of these differences. On the other hand, it is noteworthy that the bacteria constantly associated with a heterotrichous ciliate, *Parablepharisma pellitum* Kahl, are quite different and do not contain sulfur.

Remanella obtusa n. sp. (Fig. 3)

Whereas the different known species of Remanella belong to the microporal interstitial fauna, this new one was found many times in the relatively coarse sand of Nobska beach.

At first sight, *R. obtusa* recalls some species of Chlamydodon by its shape and its thigmotactic behavior. This ciliate is very flat, yellowish in color, with a brown pigmented area around the mouth. It measures about 110 micra in length and is 40 micra wide. Its anterior edge is broadly rounded.

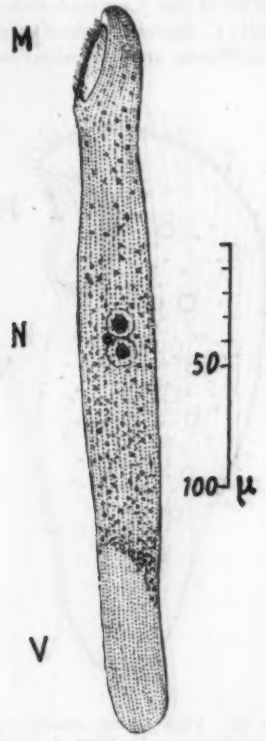


FIGURE 4. *Geleia simplex* n. sp. M, mouth; N, double macronucleus with one micronucleus; V, large posterior contractile vesicle.

The mouth appears as a lateral semi-circular notch without pharyngeal prolongation. The Müller's vesicles, with a large refringent granule in each one, are 6 to 8 in number. The animal possesses two macronuclei, each containing a central chromatic body, and a single micronucleus.

Remanella obtusa is ciliated only on its flat right side; it is very thigmotactic, and glides rapidly on the surface of the sand grains. It is a diatom feeder.

Geleia simplex n. sp. (Fig. 4)

Geleia simplex, frequently encountered in the fine sand of the Barnstable Harbor flats, differs from the other species of the genus (see Kahl, 1933, 1935; Fauré-Fremiet, 1950) by the lack of an anterior prebuccal lobe, the nearly apical location of the mouth, and the presence of a large posterior vacuole. Its length is about 260 to 300 micra, and its width about 30 micra. Its flexible and slightly contractile body is more or less flattened.

G. simplex possesses two macronuclei with large karyosomes, and one micronucleus.

The mouth is an oval depression extending anteriorly between two body rows of cilia. The buccal ciliation includes a fringe of large cilia on the right side and on the left one a field of fine undulating cilia.

This work was done at the Marine Biological Laboratory under a grant of the Commonwealth Fund, and with the aid of the American Philosophical Society. It gives me great pleasure to acknowledge here the assistance of Mr. Turner, at the Woods Hole Oceanographic Institution, in the exploration of the Barnstable Harbor flats and the measurements of some sand samples. I am grateful to Dr. M. Harnly and Dr. G. Kidder, who read the manuscript.

Summary

1. Two categories of ciliate species dwell in the damp sand of marine beaches; some of these seem to be so closely adapted to interstitial conditions that they do not live outside; some others enter the sand readily and can live in it as well as anywhere else along the seashore, where they are ordinarily widespread.

2. With regard to the size of the interstitial sand spaces, which are roughly proportional to the size of the grains, the sand-dwelling ciliates can be divided into two ecological classes:

In coarse sands, with a mean diameter of the grains above 0.4 mm., the mesoporal infusorian fauna includes a few species exclusively sand-living and many occasional sand-dwelling forms.

In fine sands, with a mean grain diameter of 0.3 to 0.7 mm., the microporal infusorian fauna is composed of true sand-living ciliate species.

In addition, some typical interstitial species, widespread in fine and coarse sands, are called euryporal.

3. Comparison of the ciliate interstitial fauna observed in Brittany and Cape Cod beaches shows that interstitial species are cosmopolitan, and that the distinguishing characteristics of mesoporal and microporal fauna have a generalized ecological significance. On the other hand, it appears that the truly microporal species are endowed with some peculiarities very interesting from the standpoint of cytology, cell-physiology and micro-ecology.

LITERATURE CITED

Calkins, G. N., 1933. The biology of the protozoa. Lea and Febiger, Philadelphia. (2nd Edition.)

FAURÉ-FREMIET, E., 1950. Ecologie des Ciliés psammophiles littoreaux. *Bull. Biol. de la France et de la Belgique*, **84**: 35–75.

KAHL, A., 1933. Ciliata libera et ectocommensalia. *In:* Grimpe und Wagler, Thierwelt der Nord- und Ostsee. Akad. Verl., Leipzig.

KAHL, A., 1935. Wimptiere oder Ciliata. *In:* Fr. Dahl, Die Tierwelt Deutschlands. Fischer, Jena.

SAUERBREY, E., 1928. Beobachtungen über einige neue oder wenig bekannte marine Ciliaten. *Arch. f. Protistenk.*, **62**: 355–407.

SPIEGEL, A., 1926. Einige neue marine Ciliaten. *Arch. f. Protistenk.*, **55**: 184–190.